FEEDBACK REGULATION AND CHARACTERIZATION OF 3-DEOXY-D-ARABINO-HEPTULOSONATE 7-PHOSPHATE SYNTHETASE IN THE MARINE ORGANISM *VIBRIO* MB22

By

ANDREW MARK CHLUDZINSKI

A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF THE UNIVERSITY OF FLORIDA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA
1972

ACKNOWLEDGEMENTS

The author wishes to express his appreciation to Dr. DeLill S. Nasser, chairman of the supervisory committee, for her encouragement, guidance, suggestions and criticisms in the conduct of the research and in the preparation of the manuscript.

He also wishes to thank Dr. Max E. Tyler, Dr. Edward P. Previc, and Dr. Eugene G. Sander for their assistance in the preparation of the manuscript.

The author also wishes to express his deepest gratitude to his wife, Peggy, for her patience and understanding and to his parents for their encouragement and assistance.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract of Dissertation Presented to the
Graduate Council of the University of Florida in Partial Fulfillment
of the Requirements for the Degree of Doctor of Philosophy

FEEDBACK REGULATION AND CHARACTERIZATION OF
3-DEOXY-D-ARABINO-HEPTULOSONATE 7-PHOSPHATE SYNTHETASE
IN THE MARINE ORGANISM VIBRIO MB22

By

Andrew Mark Chludzinski

August, 1972

Chairman: DeLill S. Nasser
Major Department: Microbiology

Crude extracts of a marine organism Vibrio MB22 chromatographed on DE-32 cellulose and hydroxylapatite have been shown to contain three separate fractions of 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthetase. Each of the fractions is inhibited primarily by one of the amino acid end products of the pathway, tyrosine, tryptophan and phenylalanine. Kinetic properties of these partially purified isozymes were studied. These isozymes demonstrate cooperative responses to varied concentrations of their respective feedback inhibitors. The substrate saturation curves for the isozymes were sigmoidal to varying degrees for 2-phosphoenolpyruvate, while only the tyrosine sensitive isozyme gave a cooperative response to varied concentrations of D-erythrose-4-phosphate. The presence of inhibitor only slightly affected these cooperative responses. The effect of inhibitors on the substrate affinity and maximum velocity of the isozymes indicates that they belong to the "V system" of allosteric enzymes. The molecular weights of the isozymes were determined by gel filtration. Catalytic activity of crude extract is stimulated 5-fold at 10^{-3} M Co^{++}. Divalent cations restore DAHP synthetase activity after treatment with EDTA in crude extract and

the tyrosine sensitive DE-32 cellulose fraction in the following order: $Co^{++} > Mn^{++} > Zn^{++} > Ni^{++} > Sn^{++} > Ca^{++}$. The tryptophan and phenylalanine sensitive fractions were primarily activated by Mn^{++} followed by Co^{++}. All three DE-32 cellulose fractions were protected from EDTA inactivation by phosphoenolpyruvate. The stimulation by divalent cations was not apparent for isozymes which were further purified by chromatography on hydroxylapatite. These isozymes were stabilized by the protein fractions removed by hydroxylapatite chromatography, as well as by phosphoenolpyruvate and to a lesser extent by bovine serum albumin. Monovalent cations had either no effect or a slight inhibitory effect on the isozymes, regardless of their state of purity.

INTRODUCTION

The living cell under a given set of extracellular conditions maintains small molecule metabolites at relatively steady state intracellular concentrations. This is a dynamic process by which the internal concentrations of metabolites depends on a balance of synthesis and utilization of these metabolites. This process is also involved in balanced distribution of available energy. Regulation therefore involves the coordination of many highly interdependent biochemical pathways to ensure an orderly supply of energy and catabolites, as well as balanced partitioning of common precursors among competing pathways in order to ensure an adequate supply of end products.

The experimental portion of this dissertation is concerned with one aspect of control, the regulation of biosynthetic enzymes' activity by the end products of the pathway. The following literature review will therefore be limited to this phenomenon of end product inhibition.

In 1941, Zacharias Dische found that mono- and diphosphoglyceric acids inhibited the phosphorylation of glucose in either intact or lysed preparations of erythrocytes (16). The inhibitors were neither substrates, products of the inhibited reaction, nor structural analogues of the substrate. Since the formation of phosphorylated glyceric acids depends on the prior phosphorylation of glucose, the final product was shown to be regulating its own rate of synthesis by directly acting on an enzyme several steps removed.

This phenomenon was examined further by many microbial physiologists

using auxotrophic mutants of bacteria (25). They observed that accumulation of precursors due to the loss of enzyme activity via mutation had a peculiar relationship to growth. It was found, using these mutants, that by supplying an intermediate of the pathway beyond the blocked reaction cells would grow, but that the time of maximum accumulation of substrate for the nonfunctional enzyme was not the time of maximal growth of the organism. In fact, substrate accumulation did not begin until the growth factor intermediate was exhausted. If the growth factor intermediate was added again, precursor production stopped. There was no further accumulation if growth stopped before the growth factor intermediate was exhausted.

This type of precursor accumulation was observed by Gots (49). Escherichia coli blocked in the synthesis of purines, in the presence of sulfonamide accumulated 4-amino-5 imidazole carboxamide, to a lesser extent in the presence of exogenous purines, than in their absence. The failure of substrate to accumulate until the exhaustion of the end product could be due to either end product control of enzyme level or function. Brooke et al. (6) found evidence supporting the latter. They found using a uracil auxotroph of Aerobacter aerogenes blocked after the synthesis of orotic acid, that orotic acid did not accumulate in the presence of uracil but accumulated starting immediately after cells were transferred to uracil-free medium. Thus, uracil when present controlled the function of the enzymatic sequence.

Similar conclusions were reached by Roberts et al. (113) using a prototrophic Escherichia coli. They tested the ability of a compound to compete with a general carbon source, such as glucose, in the biosynthesis of small molecule building blocks. The cells used for inoculation of cultures were harvested during the logarithmic phase of

growth in a minimal medium. Therefore, it was assumed that these cells had all the enzymes required for the synthesis of cellular amino acids. When the fresh medium contained threonine in addition to radioactive glucose, no incorporation of label into cellular threonine was observed. Since the enzymes involved were present, it could only be concluded that threonine exerted some effect either directly or indirectly on the biosynthetic sequence leading to threonine production.

Umbarger (144) proved that isoleucine directly controlled threonine deamination by the use of cell free extracts of E. coli which contained threonine deaminase activity. This enzyme was shown to be completely inhibited in the presence of isoleucine in vitro. This inhibition was specific for isoleucine in that other amino acids showed little or no inhibitory effect.

Yates and Pardee (160) using crude extracts of E. coli showed that cytidylic acid inhibits the activity of the enzyme catalyzing the carbamylation of aspartate in the pyrimidine biosynthetic pathway. This work corroborated the earlier work of Brooke et al. (6), with pyrimidine auxotrophs. The same pattern of control was observed by Strecker (135) in the proline biosynthetic pathway.

In each of the above cases, the feedback loop involved the end product of the biosynthetic sequence and what presumably was the first enzyme leading specifically to that end product. This hypothesis was tested by Umbarger and Brown (150) in the valine biosynthetic pathway of E. coli. They found that valine does inhibit the production of α-acetolactate from pyruvate, the first reaction of the pathway. Umbarger (145) proposed that the phrase "end-product inhibition" be employed to describe this mechanism of control. Soon after this early work many

other examples of this type of control were reported in many different biosynthetic pathways (5, 16, 115, 127, 145, 146, 147).

Feedback regulation of unbranched biosynthetic pathways is relatively simple because the pathway only produces one end product, which can be an inhibitor, as in histidine biosynthesis (1, 86). However, regulation of a branched biosynthetic pathway with two or more end products is more complicated. Since the precursors are common to all the end products, independent control by an excess of any one of them could cause a deficiency in the production of the other end products (16, 126).

The control of enzyme function in branched pathways has been found to be achieved by a number of different mechanisms. The type of control utilized may vary for the same pathway in different organisms. Some types of feedback mechanisms which have been described are as follows: Enzymes with multiple inhibitor sites. a) Concerted or multivalent feedback inhibition occurs when neither end product alone is able to inhibit the first enzyme of the pathway, but when both are in excess simultaneously the enzyme is inhibited. This inhibition of the flow of metabolites is reinforced by the feedback inhibition of the first reaction of each branch by its specific end product. This mechanism has been found for aspartokinase in Rhodopseudomonas capsulata (23) and Bacillus polymyxa (100). b) Synergistic feedback inhibition differs from concerted only in that each end product has a slight ability to act independently of the other. However, when both are in excess simultaneously the inhibition is greater than the sum of their independent inhibitions. This was first observed in purine nucleotide biosynthesis in Aerobacter aerogenes and pigeon liver (12, 96). This mode of in-

hibition has been called cooperative feedback inhibition. Since it might be confused with cooperativity of allosteric enzymes, the term synergistic is preferred. Glutamine synthetase in Bacillus licheniformis (57) and aspartokinase in E. coli (69, 142) are also subject to this form of control. c) In cumulative feedback inhibition, each end product acts independently of the others and causes only a partial inhibition of the first reaction of the pathway. It differs from synergistic inhibition in that the inhibition caused by mixtures of end products is quantitatively determined by the capacity of each inhibitor by itself to inhibit the enzyme. The enzyme activity remaining in the presence of more than one end product is equal to the product of the fractional activities observed for each inhibitor alone. This was first observed for glutamine synthetase in E. coli by Woolfolk and Stadtman (157, 158). Again, this inhibition of the flow of metabolites is reinforced by specific end product control of the first divergent step in each branch of the pathway. The significance of this type of control for this enzyme has also been shown to be dependent on whether the enzyme is adenylated or unadenylated (64, 118). d) In compensatory antagonism of end product inhibition, an enzyme is conditionally inhibited by an end product. Uridylic acid is found in E. coli to be capable of completely inhibiting carbamyl phosphate synthetase, but in the presence of ornithine, maximum inhibition is only 60% (103). The level of ornithine is controlled by arginine. This enzyme is therefore indirectly under concerted control of uridylic acid and arginine. Valine is a compensatory antagonist of isoleucine inhibition of threonine deaminase in Bacillus subtilis (147). e) Sequential feedback control was first shown by Nester and Jensen (94) in aromatic amino acid biosynthesis in

B. subtilis. Here, the first enzyme of the common pathway is sensitive to the last common intermediate in the branched pathway, but not the end products. In addition, the first step of each divergent pathway is under feedback control of the ultimate end product unique to that part of the total pathway. Sequential feedback control has also been found for threonine and isoleucine biosynthesis in Rhodopseudomonas spheroides (21, 22, 24). f) Enzyme multiplicity involves at least two enzymes which catalyze the first step of the common pathway, but which respond to different effectors. Excess of one end product will inhibit only one of the catalytically identical enzymes. Thus synthesis continues at a reduced rate via the remaining enzymes.

This type of control mechanism is operative in a number of biosynthetic pathways. Three aspartokinases and two homoserine dehydrogenases in E. coli are involved in control of lysine, threonine, methionine and isoleucine biosynthesis (17, 99, 129). There are multiple forms for the first biosynthetic enzyme of the aromatic amino acid pathway in E. coli (7, 8, 35, 123) and A. aerogenes (8). The latter also contains two chorismate mutases (19). Saccharomyces cerevisiae has two carbamyl phosphate synthetases, the enzyme in both pyrimidine nucloetide and arginine biosynthesis (68).

This type of regulation is not restricted to branched biosynthetic pathways. It may also be utilized when a given reaction is required for two or more functions as in the conversion of pyrrolidine-5-carboxylate to glutamic acid, a common step for degradation of arginine and proline (26). Multiple forms of enzymes are also known to catalyze a step common to both a biosynthetic and degradative pathway. Two threonine deaminoses in E. coli are known to catalyze conversion of

threonine to α-ketobutyrate, the first step in biosynthesis of isoleucine and the first step in the degradation of threonine when it is supplied under anaerobic conditions (144, 147, 148). Two α-acetolactate synthetases in A. aerogenes are concerned with valine biosynthesis and fermentative metabolism of pyruvate at low pH (147, 149). Another example is the presence of two ornithine transcarbamylases in some pseudomonads (110, 130) which are involved in the biosynthesis or fermentation of arginine.

The biosynthesis of the aromatic amino acids is accomplished by a branched pathway which produces tyrosine, tryptophan, phenylalanine and vitamins as its end products. Animals are incapable of synthesizing benzene rings but microorganisms and plants may form aromatic compounds by several pathways. The "shikimate" pathway (46) is important in all microorganisms while the "acetate" pathway operates mainly in fungi and plants which are therefore capable of forming a wide variety of aromatic compounds (43, 111). We will concern ourselves with the former pathway. This pathway involves the condensation of two products of carbohydrate metabolism, erythrose-4-phosphate (E-4-P) and phosphoenolpyruvate (PEP) to a seven-carbon compound, 3-deoxy-D-arabino-heptulosonate (DAHP) (32,124). This compound is further converted to shikimate and finally to chorismate, the last common intermediate for all of the end products. At this point, the "shikimate" pathway branches and culminates in the formation of tyrosine, tryptophan, phenylalanine and "vitamins" (Figure 1) at least in E. coli and A. aerogenes (32, 46, 70, 105, 151), via individual branches.

The first reaction of the pathway is catalyzed by 3-deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthetase (E.C. 4.1.2.15). As the first enzyme of a complex metabolic pathway, it is subject to rigorous control.

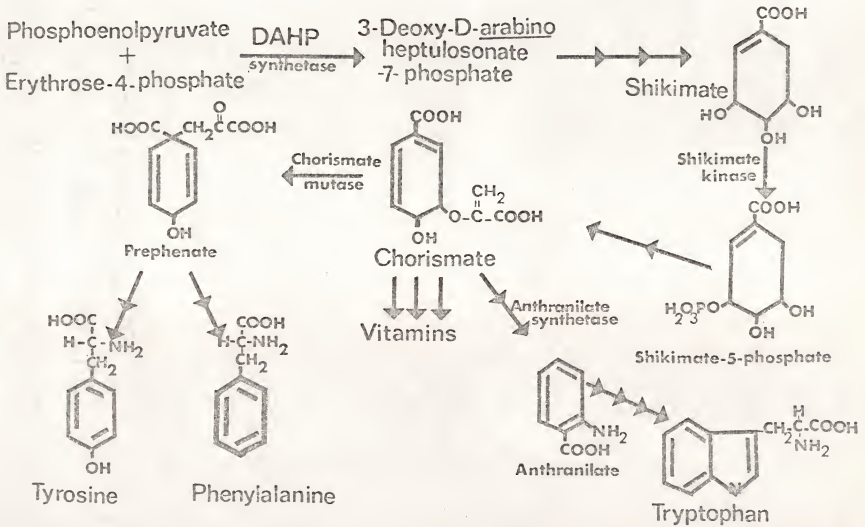

Figure 1. Pathway for the biosynthesis of the aromatic amino acids.

Many microorganisms have been demonstrated to have different modes of feedback regulation at this point in the pathway (59).

Feedback inhibition of DAHP synthetase was first described by Smith et al. (123). They showed that it was possible to inhibit this enzyme activity in crude cell free extracts of E. coli W with either phenylalanine or tyrosine. Ammonium sulfate fractionation separated two enzyme activities, one inhibited by phenylalanine, the other by tyrosine. However, tryptophan did not inhibit any DAHP synthetase activity. Brown and Doy (7) demonstrated in E. coli W the existence of a third isozyme by varying growth conditions of this organism. They found that cells grown in the presence of L-phenylalanine and L-tyrosine had DAHP synthetase activity which was not inhibited by either of these amino acids. This type of activity was enhanced when appropriate mutants were limited for L-tryptophan. The presence of L-tryptophan in the medium repressed the synthesis of this non-inhibitable enzyme. These workers also found an ammonium sulfate fraction of crude extract, which contained the DAHP synthetase activity, that was not inhibited by any of the end products but was repressed by tryptophan in vivo (35).

Wallace and Pittard (152, 153) using DEAE-cellulose chromatography showed that three enzymes of DAHP synthetase exist in E. coli K12. Each of these isozymes was responsive to one of the aromatic amino acids. However, the tryptophan isozyme could only be repressed by L-tryptophan and not inhibited. Brown and Maas (9) also obtained similar results with E. coli K12. Doy (29) demonstrated a 32% inhibition of DAHP synthetase activity by tryptophan in extracts of E. coli W tryptophan auxotrophs grown under conditions of limiting tryptophan. Pittard et al.

(104) found that growth of a strain of E. coli K12 which possessed only the single isozyme of DAHP synthetase (tryp) and which synthesizes this enzyme constitutively is inhibited by tryptophan. The accumulation of DAHP by a derivative of this strain unable to convert DAHP to dehydroquinate is also inhibited by tryptophan. This isozyme was much more sensitive to tryptophan in the presence of Co^{++}.

Salmonella typhimurium also contains isozymes of DAHP synthetase. DAHP synthetase activity in crude extracts was additively inhibited by phenylalanine and tyrosine. Ammonium sulfate fractionation of the crude extract separated both phenylalanine and tyrosine sensitive activities of DAHP synthetase (47, 58, 59). Gibson and Gibson (45) isolated mutants which lacked, individually, DAHP synthetase isozymes.

Saccharomyces cerevisiae appears to have only two DAHP synthetase isozymes, one inhibited by tyrosine and the other by phenylalanine (31, 71, 72). Mutant strains, each lacking one of the isozymes, have been isolated (88). A recombinant strain derived from the two strains has no detectable DAHP synthetase activity and is unable to grow in minimal medium. Tryptophan, however, has its earliest point of control at anthranilate synthetase (36).

In Neurospora crassa there are three isozymes, each inhibited by one of the aromatic amino acids (29, 30, 52, 58). Gel filtration of N. crassa cell-free extracts has separated the three DAHP synthetase isozymes (34, 58). Mutants lacking each of the isozyme activities have been isolated, as well as recombinant strains each containing only one isozyme activity. The recombinant strains have been used to find feedback resistant mutants for each isozyme (51, 52). Recent work has shown that both phenylalanine and tyrosine inhibitable isozymes

dissociate into half molecules in the presence of their inhibitors, while the feedback resistant isozymes show no change in molecular weight in the presence of these compounds (34).

Claviceps paspali (73) and SD58 (41) have also been demonstrated to have three isozymes each inhibitable by one of the aromatic amino acids.

Weber and Böck (155) found only one DAHP synthetase activity for the green algae Euglena gracilis. Both tyrosine and phenylalanine were found to inhibit this enzyme, most likely at the same site.

Streptomyces aureofaciens Tü24 has recently been shown (48) to possess a single DAHP synthetase enzyme, sensitive only to L-tryptophan.

Investigation of B. subtilis has shown that DAHP synthetase is controlled by sequential feedback inhibition (60, 61). Jensen and Nester (61) proved that there is only a single enzyme by means of gel filtration, DEAE-cellulose chromatography, ammonium sulfate fractionation and single step mutants. This activity can be inhibited to a maximum of 80% by either prephenate or chorismate, while the aromatic amino acids have no inhibitory effect. Each aromatic amino acid does, however, inhibit the first enzyme of its particular branch. In this manner, the concentration of prephenate would reflect the available levels of phenylalanine and tyrosine, while the concentration of chorismate would reflect the available level of tryptophan (94). Inhibition by chorismate and prephenate was found to be neither synergistic nor additive, indicating possibly one site or interacting sites for both effectors. Chorismate and prephenate also feedback inhibit an enzyme intermediate in the pathway, shikimate kinase. This enzyme, as well as chorismate mutase and DAHP synthetase, appears to be aggregated in this organism (74, 95).

A survey of the mode of control of DAHP synthetase in various organisms (59) showed that closely related species tended to have the same regulatory mechanism for DAHP synthetase. Six distinct patterns of feedback control of DAHP synthetase were demonstrated in microorganisms surveyed.

Although branched biosynthetic pathways in terrestial organisms have been shown to be regulated by various feedback control mechanisms, this has not been studied to any extent in marine organisms. We therefore initiated this study.

Marine bacteria are bacteria which require for growth a medium made with sea water. This medium must contain, as does natural sea water, the major ionic components Na^+, K^+, Mg^{++}, Ca^{++}, Cl^- and $SO_4^=$ (55). The uniqueness or lack of uniqueness of marine bacteria has been discussed in a review by MacLeod (76).

Taxonomically, marine bacteria do not appear to be unique. They can be classified in a defined group of non-marine organisms (119). However, they possess a few characteristics which distinguish them from non-marine organisms.

All organisms of a marine origin requiring sea water for growth have a stable, irreplaceable minimum requirement for Na^+ (82, 107, 108, 143, 161), but vary in the Na^+ concentrations they require for optimal growth (107, 108, 120, 143). Marine organisms also have a specific requirement for other ionic constituents of sea water at higher concentrations than do non-marine organisms (82, 84).

The specific Na^+ and K^+ requirement for growth of a marine pseudomonad and Photobacterium fischeri was found to be the same for the oxidation of substrates (83, 102, 108, 138). The Na^+ requirement for

the uptake of labelled L-arabinose, mannitol, glucuronate and lactose by Pseudomonas natriegens, a marine pseudomonad, was believed to be necessary for activation of the permease and catabolism systems (101, 102, 112). This failed to account for the Na^+ requirement for oxidation of succinate, citrate and fumarate by marine pseudomonads which have constitutive permeases (77, 102, 138).

Drapeau and MacLeod (38, 39) using Photobacterium fischeri and a marine pseudomonad demonstrated that the Na^+ requirement for oxidation of substrates reflected a specific need of the cell for the transport of substrates. Experiments with L-alanine, D-galactose and their non-metabolizable analogues α-aminobutyrate-C^{14} and D-fucose-H^3 showed that the level of Na^+ required for transport was dependent on the type of substrate being oxidized. Vibrio natriegens has also been shown to require Na^+ and K^+ for mannitol-C^{14} transport (154). The Na^+ requirement for retention of substrates is not as specific as for uptake (136, 159).

It is now believed that the Na^+ requirement for transport and retention of solutes is manifested at the cytoplasmic membrane, since spheroplasts of a marine organism require Na^+ for α-aminobutyrate-C^{14} uptake and retention (18, 28). Thompson and MacLeod (140) believe that the role of sodium was to increase the affinity of a carrier protein for the molecule to be transported. Once the substrate is carried across the membrane K^+ and ATP reduce this affinity and the substrate accumulates. So K^+ may be a cofactor in the coupling of phosphate bond energy or a high energy intermediate to the transport system (87).

The Na^+ requirement for growth is not unique for marine organisms. There have been many reports on non-marine organisms which have a

specific need for Na^+ in normal metabolism but at much lower concentrations (20, 67, 98, 122, 134, 156). The requirement for K^+ and/or a high ionic strength by an enzyme again is not restricted to marine organisms (53, 97).

Metabolic pathways studied in marine organisms are similar to those found in non-marine organisms. P. natriegens dissimilates glucose by the glycolytic and pentose monophosphate pathways and gluconate by the Entner-Douderoff pathway (40, 112). MacLeod et al. (77, 78, 79) have demonstrated the glyoxylate and TCA cycle in a marine Pseudomonas and Spirillum.

MacLeod et al. (78) found that in cell-free extracts of marine Pseudomonas dehydrogenase, fumarase and α-ketoglutarate dehydrogenase were inactivated by Na^+ and K^+, while the condensing enzyme was not affected by either ion. Malic dehydrogenase and acetate activating enzyme required K^+. However, isocitrate dehydrogenase and aconitase were most active at ionic strengths of 0.2 to 0.4 m, but were not affected by specific ions (77, 78). Tryptophanase from a marine Vibrio also has a K^+ requirement in cell free extracts (109). Thompson et al. (139) have shown that a cell envelope nucleotidase of a marine bacterium MB3 specifically required Mg^{++} and can be stimulated further by monovalent cations. Non-marine and mammalian cells also have this requirement but at a much lower concentration (10, 50). This requirement by marine organisms may reflect the need of the outer cell layers for an optimum cationic environment, which may be related to enzyme activity. This enzyme differs from those in mammalian cells and non-marine organisms in that it can hydrolyze AMP and ADP rather than just ATP. These ion requirements have also been shown in V. parahaemolyticus (56)

and a marine bacterium (37). Whether such an ATP-hydroalyzing enzyme is characteristic and unique to marine bacteria has yet to be shown.

Much of the reported data dealing with regulation in marine organisms is concerned with induction of permeases and oxidative enzymes. These enzymes require Na^+ for induction as shown for a marine pseudomonad by glucuronate (101, 102), P. natriegens by glucuronate, L-arabinose, mannitol and lactose (112) and V. natriegens by L-mannitol (154). Luciferase is known to be induced by L-arginine in Achromobacter fischeri (11). Eagon and Wong (40) have demonstrated gluconate induction of enzymes found in the Entner-Douderoff pathway. Enzymes of the glyoxylate cycle, isocitrate and malate synthetase are only present when acetate is the sole carbon and energy source in a marine Pseudomonas (79). Feedback inhibition has been observed for alkaline phosphatase by low concentrations of orthophosphate (106). This is similar to results obtained with this enzyme from E. coli (141).

One of the major properties found for all marine bacteria is lysis when suspended in distilled water or dilute buffer (54, 63, 108, 143). MacLeod et al. (80, 81) observed that Mg^{++} and other divalent cations prevented lysis, although the NaCl concentration necessary to protect against lysis varied among species tested. At present it is believed that Mg^{++} bridges anionic groups of the cell wall subunits and Na^+ can protect cell walls by screening these negative charges (18, 27, 28, 42).

Sea water has been shown to have a bactericidal effect on non-marine organisms which cannot be attributed to just salt effects. This bactericidal activity of natural and artificial sea water has been demonstrated with E. coli (117). The effect was believed to be due to the presence of toxic heavy trace metals. Staphylococcus aureus but

not E. coli has been shown to be affected by a non-dialyzable, heat labile compound in sea water (116).

The mechanisms which confer on the bacteria the capacity to survive and grow in sea water may be important in distinguishing marine and non-marine organisms (76).

MATERIALS AND METHODS

Organism

The marine bacterium used in this work, Vibrio MB22 was obtained from Dr. M. E. Tyler of this department. The isolation and characterization of this organism has been previously described (143).

Media

Artificial sea water (ASW) was used for washing cells as well as for complex medium preparation. It consisted of the following salts: 0.4 M NaCl, 10 mM KCl, 26 mM $MgCl_2 \cdot 6H_2O$ and 28 mM $MgSO_4 \cdot 7H_2O$. The complex medium (TASW) consisted of 1% trypticase (Baltimore Biological Lab Inc., Baltimore, Md.) solubilized in full strength ASW. Cultures were checked for non-marine contaminants by growth on 1% trypticase, distilled water medium. Solid medium was made by adding 2% Bacto Agar (Difco Laboratories, Detroit, Mich.) to the complex broth. The pH after sterilization was 6.8.

The minimal medium (D5) used in this work, consisted of 0.27 M Na Cl, 9.4 mM KCl, 0.17 mM $MgCl_2 \cdot 6H_2O$, 0.2 mM $MgSO_4 \cdot 7H_2O$, 37 mM NH_4Cl, 6.5 mM $NaH_2PO_4 \cdot H_2O$, 25 mM Na_2HPO_4 and 23 mM sodium gluconate. The phosphates, the carbon source, and the remaining inorganic salts were autoclaved in separate portions and mixed together after cooling. The final pH was 7.2. When the D5 medium was used in solid form, 1% lonagar (Colab, Chicago Heights, Ill.) was added.

Culture Growth

The organism was routinely cultured on TASW and D5 agar. Cultures were checked for contamination by streaking TASW, TD and D5 media. Primary stocks were maintained in TASW agar under oil and as lyophilyzed cultures.

Routinely, about 2 ml of TASW medium were inoculated from a single colony of Vibrio MB22 grown on solid D5 medium and the cells were grown at 27 C, with aeration, for about 12 hrs. The culture was centrifuged and the cells were resuspended in 25 ml of D5 medium. Growth was continued at 27 C with shaking for about 16 hrs. Two hundred ml of D5 medium in 1L erlenmeyer flasks were then inoculated with 5 ml of this culture. These cultures were grown under the same conditions to a Klett (Klett-Summerson Colorimeter, Klett Instrument Co., New York, N. Y.) reading of about 160 (filter #66) or approximately 5×10^9 cells per ml, corresponding to the middle of the exponential phase of growth of this organism under these conditions. The cells were then harvested and washed with artificial sea water by centrifugation in a RC-2-B centrifuge (Ivan Sorvall Inc., Norwalk, Conn.) using a GSA rotor at 10,400 x g for 10 minutes at 4 C. The pellet was resuspended in 20 ml ASW and centrifuged in a SS-34 rotor at 27,000 x g for 10 minutes at 4 C. This pellet was used for preparation of extracts.

Large amounts of cells were prepared by inoculating 13 liters of D5 medium with 600 ml of inoculum prepared as described in the previous paragraph. The culture was grown in a Microferm Fermentor (New Brunswick Scientific Co., New Brunswick, N. J.) with aeration at 27 C. In this case, the cells were harvested with a Delaval cream separator (Delaval Separator Co., Poughkeepsie, N. Y.) at 4 C. Approximately 5 g

(wet weight) of cells per liter of medium were obtained. The cells were washed with artificial sea water as previously described and resuspended in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Portions were frozen at -70 C. Cells frozen under these conditions did not exhibit any change in either level of 3-deoxy-D-arabino heptulosonate 7-phosphate (DAHP) synthetase activity or in the pattern of inhibition.

Extract Preparation

Cells were suspended in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$ and broken in a French Pressure Cell (American Instrument Co., Silver Spring, Md.) at approximately 8,000 psi. Cellular debris was removed by centrifugation at 27,000 x g for 30 minutes. Nucleic acids were removed from the supernatant by adding 0.1 ml of a 1.5% protamine sulfate solution per 10 mg protein in the supernatant. The protamine sulfate was added dropwise with stirring at 4 C. Stirring continued for 30 minutes after all the protamine sulfate was added. The precipitate was removed by centrifugation at 27,000 x g for 30 minutes at 4 C. Unless otherwise noted, this supernatant is called the crude extract and contained about 14 mg/ml protein.

Enzyme Assay

The assay for DAHP synthetase was done according to the method described by Srinivasen et al. (125) as modified by Jensen and Nester (60). The reaction mixture contained 20 µmoles potassium phosphate buffer, pH 6.5; 0.4 µmoles of erythrose-4-phosphate; and 0.57 µmoles phosphoenolpyruvate in a total volume of 0.20 ml. The enzyme preparation (0.05 ml) was added to 0.15 ml of reaction mixture at 4 C. The reaction

was initiated by placing the tubes in a 37 C water bath. The reaction mixture was incubated for 10 minutes. The rate of DAHP formation was determined to be linear with the time of the assay and concentration of the extract. The reaction was terminated with 0.04 ml of trichloroacetic acid and the protein was sedimented by centrifugation. The supernatant (0.1 ml) was added to 0.1 ml of 0.025 M periodate in 0.125 N H_2SO_4 and incubated at 37 C for 30 minutes. At the end of this period 0.2 ml of 2% sodium arsenite in 0.5 N HCl was added followed by 0.8 ml of 0.3% thiobarbituric acid. Color was developed by heating at 100 C in marble capped tubes for 10 minutes and the absorbance was read at 549 nm with a Gilford Model 240 Spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio). Controls were included in order to correct for any non-specific absorption at 549 nm. Inhibition of color formation by all compounds used was also determined. The amount of DAHP was calculated with the use of the value $4.5 \times 10^4 M^{-1} cm^{-1}$ for the molar extinction coefficient at 549 nm. Specific activity is defined as mμmoles DAHP produced/min/mg protein.

Effector Assay

Inhibitors were tested by adding a volume of the compound to be tested to an equal volume of either crude extract or column effluents. The compounds were dissolved in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$, to a concentration twice the final concentration recorded in the tables. The mixtures of inhibitor and extract were incubated at 4 C for 10 min before addition to the reaction mixture. Monovalent and divalent cations were made up in 0.05 M potassium phosphate buffer, pH 7.0 and added to the extract to the concentration

recorded in the tables. Ethylenediaminetetraacetic acid was added to a final concentration of 10^{-4} M and incubated for 10 minutes at 4 C.

pH Effect

Isozyme activities were diluted in 0.2 M buffer at the specified pH, incubated 10 minutes at 4 C and then assayed by the standard method. All buffers contained 0.1 mM $CoCl_2$. The following buffers were used: citrate-potassium phosphate, pH 4.8 to 5.6; potassium phosphate pH 6.0 to 8.0; glycine-sodium hydroxide, pH 9.0 to 10.0.

Protein Determinations

Protein was determined by the method of Lowry et al. (75). Crystalline bovine serum albumin (Pentex, Inc., Kankakee, Ill.) was used as the standard protein. Protein eluted from columns was estimated by determining absorbancy of the fractions at 280 nm in a Gilford Model 240 Spectrophotometer (Gilford Instrument Laboratories Inc., Oberlin, Ohio).

DEAE-Cellulose Chromotography

Diethylaminoethyl (DEAE) cellulose (Whatman DE-32, 1.0 meq/ml, Reeve Angel, Clifton, N. J.) was prepared as recommended by the manufacturer and packed into columns of the sizes indicated in the specific experiments. All columns were equilibrated with the appropriate buffer before use. Protein was eluted from the columns by a linear gradient of KCl made up in the same buffer. The average flow rates and the volume of the fractions collected are indicated in the specific experiments.

Hydroxylapatite Chromatography

Hydroxylapatite was prepared according to the procedures of Main et al. (85), equilibrated in 0.05 M potassium phosphate buffer, pH 6.9 + 0.1 mM $CoCl_2$ and packed into a column (1.5 x 25 cm, 16 ml void volume). Protein was eluted from the column by a linear gradient of 0.05 to 0.3 M potassium phosphate buffer, pH 6.9, + 0.1 mM $CoCl_2$. Flow rate of 20 ml/hr was maintained with a Sigma pump Model A1-2E-34 (Sigma Motors, Inc., Middleport, N. Y.) and fractions of 2.1 ml were collected.

Protein Concentration

When DAHP synthetase activities were separated from each other on one column and pooled tubes of effluent were loaded onto another column, the protein was first concentrated by the use of solid $(NH_4)_2SO_4$ (enzyme grade) or dialysis against polyethylene glycol. $(NH_4)_2SO_4$ was added slowly to 75% saturation at 4 C and after 30 minutes of stirring at the same temperature, the precipitated protein was removed by centrifugation at 27,000 x g for 20 min. The precipitate was resuspended in 5 ml of buffer and passed through a Sephadex G-25 column to remove the $(NH_4)_2SO_4$. Alternately, in some cases the protein was concentrated by dialysis of the pooled effluents against 25% polyethylene glycol in 0.05 M potassium phosphate buffer pH 7.0 + 0.1 mM $CoCl_2$ at 4 C for 5 hr. The pooled volume was concentrated approximately 10- to 15-fold.

Ammonium Sulfate Fractionation

Extracts were fractionated with $(NH_4)_2SO_4$ (enzyme grade) according to the method of Doy and Brown (35). Each fraction was solubilized in

2 ml of the appropriate buffer and passed through a Sephadex G-25 column to remove the $(NH_4)_2SO_4$.

Gel Filtration

A Sephadex (Pharmacia Fine Chemicals, Piscataway, N. J.) G-25 column (1.5 x 25 cm, 16 ml void volume) was prepared as suggested by the manufacturer. A maximum of 3 ml was passed through the column at a flow rate of approximately 60 ml/hr. One ml fractions were collected. Fractions containing the most protein as estimated by absorbancy at 280 nm were combined.

Gel filtration on Sephadex G-200 (3) was used to determine the molecular weight of DAHP synthetase isozymes. The column (2.5 x 45 cm, void volume = 74 ml) was prepared according to the procedure of Andrews (2). The column was equilibrated with 0.05 m potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. The column was eluted by reverse flow at a rate of 12 ml/hr. All standards were added in 2 ml volumes of buffer and 2 ml fractions were collected.

Bovine liver catalase, molecular weight 252,000,was assayed by measurement of the disappearance of H_2O_2 at 240 nm (13). Rabbit muscle lactate dehydrogenase, molecular weight 136,000, was assayed by the method of Kornberg (65). Rabbit α-globulin, molecular weight 160,000, bovine serum albumin, molecular weight 67,000, ovalbumin, molecular weight, 45,000, and cytochrome C, molecular weight 12,270,were determined by absorbance at 230 nm. DAHP isozymes were run individually in 2 ml volumes (10 to 30 mg protein). The void volume was determined with blue dextran.

Cell Hydrolysis

Cells of Vibrio MB22, grown in D5 medium containing either C^{14}-phenylalanine or C^{14}-tyrosine, were harvested, washed with artificial sea water and resuspended in distilled water. They were freeze-dried in a Virtis freeze-dry unit (Virtis Research Equipment Co., Gardiner, N. Y.) for a minimum of 5 hrs and then resuspended in 2 ml of 6N HCl. This suspension was sealed in a screw cap tube and the cells were hydrolyzed for 24 hr at 100 C. The hydrolysate was evaporated to dryness, and dehydrated six times. It was then resuspended in distilled water, debris removed by centrifugation, and filtered through 0.8 μ Millipore filter (Millipore Corp., Bedford, Mass.).

Thin Layer Chromatography

Two-dimensional chromatography was done on 20 x 20 cm I.T.L.C., type SG chromatography medium (Gelman Instrument Co., Ann Arbor, Mich.). The first phase was n-propanol: conc. ammonia (70:30); the second phase was phenol: water (100:39 w/v). The chromatograms were spotted with either 10 μl of the cell hydrolysate or the standard solutions of the known amino acids prepared at concentration of 1 mg/ml. The amino acids were visualized by spraying with 0.3% ninhydrin in acetone. Unsprayed chromatograms were examined for radioactivity. Several control amino acids as well as phenylalanine, tyrosine, and blank areas were cut out, dried and suspended in scintillation vials with 10 ml of scintillation fluid (Omnifluor, New England Nuclear, Boston, Mass.) and counted in a Tri-Carb Liquid Scintillation Counter (Packard Instrument Co., Inc., LaGrange, Ill.).

Chemicals

D-Erythrose-4-phosphate (sodium salt) as well as the aromatic amino acid L-isomers, p-aminobenzoic acid (PABA) and shikimic acid were purchased from Sigma Chemical Co., St. Louis, Mo. 2-Phosphoenolpyruvic acid (trisodium salt, pentahydrate) and protamine sulfate (salmine) were purchased from Calbiochem, Los Angeles, Calif. Enzyme grade $(NH_4)_2SO_4$ as well as sodium gluconate was obtained from Nutritional Biochemical Corp., Cleveland, Ohio. All the salts and EDTA used were obtained from J. T. Baker Chemical Corp. Radioactive phenylalanine and tyrosine were obtained from New England Nuclear Corp., Boston, Mass.

RESULTS

Although only a few species of true bacteria have been shown to be able to interconvert phenylalanine and tyrosine (14), it was considered essential to the interpretation of data concerning regulation to know whether Vibrio MB22 had this capability. Cells were grown in the presence of 2 μ curies of either tyrosine -C^{14} or phenylalanine -C^{14} (generally labelled), harvested, washed and hydrolyzed as described in Materials and Methods. The hydrolysates were analyzed by two-dimensional thin layer chromatography, using known L-phenylalanine and L-tyrosine as standards. The areas identified as L-tyrosine and L-phenylalanine, as well as other areas of the chromatogram were cut out and the amount of radioactivity localized within these areas determined. Vibrio MB 22, it was concluded, cannot interconvert phenylalanine and tyrosine (Table 1).

The type of feedback control utilized by this microorganism for DAHP synthetase was determined by analysis of activity found in crude extract. Intermediates, as well as end products of this pathway were tested for their ability to inhibit DAHP synthetase activity. Only the end products, tyrosine, tryptophan and phenylalanine inhibited enzymatic activity to significant extents at each concentration tested (Table 2). This indicated that only the amino acid end products of this branched chain pathway can act as feedback inhibitors for DAHP synthetase from this organism.

Table 1. Interconversion of phenylalanine and tyrosine by Vibrio MB22

Cells grown in the presence of	Amino acid counted	CPM
PHE -C^{14}	PHE	2,115
	TYRO	73*
TYRO -C^{14}	PHE	3
	TYRO	1,020

Vibrio MB22 was grown in D5 medium in the presence of 2 μC of either tyrosine-C^{14} or phenylalanine-C^{14}. The cells were harvested, washed and hydrolyzed. Ten μl of each hydrolysate were spotted on thin layer medium. The amino acids were visualized by spraying with ninhydrin and identified by co-chromatography with known compounds. Areas of 2 x 2.5 cm were cut out in the regions of phenylalanine and tyrosine, put into 10 ml scintillation counting fluid and counted in a Tri-Carb Liquid Scintillation Counter All procedures used are more completely described in Materials and Methods.

*The counts for tyrosine when cells were grown in phenylalanine were typically higher since, in this system, tyrosine trails phenlalanine.

Table 2. Possible inhibitors of DAHP synthetase activity from *Vibrio* MB22

Compound	Percent inhibition at final concentrations of	
	6×10^{-5} M	6×10^{-4} M
Chorismate	1	5
Anthranilate	1	10
Shikimate	4	0
Indole	0	11
para-Amino benzoate	0	0
Tyrosine	44	70
Tryptophan	27	28
Phenylalanine	5	14
Aromatic Amino Acids	70	100

Extracts were made from two separate cultures of *Vibrio* MB22 as described in Materials and Methods. The above results are the average values of separate experiments using the different extracts.

Prior to a more extensive study of the inhibition properties of this enzyme, some parameters for extract preparation were determined. The relationship of time of cell harvest to enzyme activity was examined (Figure 2). The specific activities of DAHP synthetase from cells harvested at different times in exponential growth indicated that the highest specific activity of the enzyme was seen when cells were harvested at a turbidity of approximately Klett 160 (#66 filter).

Different methods of cell breakage were also investigated (Table 3). The French pressure cell was found to give extracts with the highest specific activity for DAHP synthetase, as well as the least variability of inhibition patterns for the enzyme. Sonication was found to produce enzyme activities which varied in their sensitivity to inhibitors, apparently due to desensitization of the enzyme to different degrees with different preparations.

The effect of protamine sulfate treatment of crude extracts on the activity and feedback inhibition patterns of DAHP synthetase was investigated next. Crude extracts were prepared with various concentrations of protamine sulfate and these parameters were measured (Table 4). Treatment with 1.5% protamine sulfate gave the highest specific activity and showed some inhibition of enzyme activity by phenylalanine.

Previous work (92, 114) with crude extract of this organism had showed that $CoCl_2$ stimulated DAHP synthetase activity. This stimulation was reproduced in my work and, therefore, $CoCl_2$ was included in all buffers at a final concentration of 0.1 mM. However, NaF, which was thought to activate the enzyme (114), was found to actually inhibit activity of DAHP synthetase (Table 5). Extracts prepared in these various buffers showed that the highest specific activity of DAHP syn-

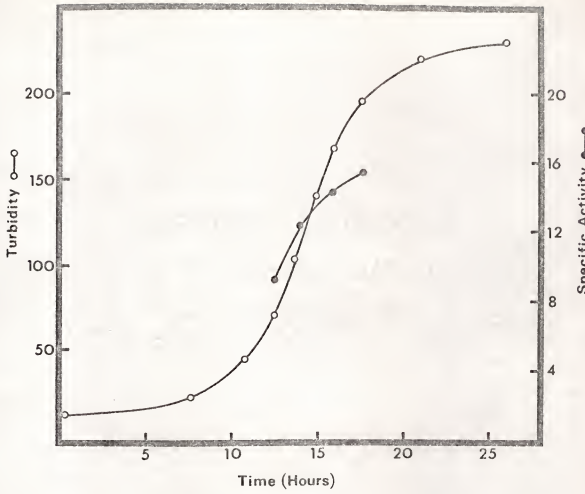

Figure 2. Activity of DAHP synthetase from cells harvested at different stages of growth. Cells were grown in D_5 medium with aeration at 27 C. Growth was monitored by following absorbance with a Klett-Summerson colorimeter (#66 filter). At the designated times a 200 ml aliquot of culture was removed and crude extract was prepared as described in the text. Specific activity is defined as mμ M DAHP produced/mg protein/minute.

Table 3. Specific activity and feedback inhibition of crude extracts prepared by different methods of cell breakage

Method	Specific Activity	Effector	Percent Inhibition
Freeze-Thaw	14	TYRO	12
		TRYP	21
		PHE	9
		ARO*	36
Sonication	24	TYRO	4
		TRYP	9
		PHE	34
		ARO	34
French Press	30	TYRO	30
		TRYP	21
		PHE	9
		ARO	50

Extracts were not treated with protamine sulfate. Effectors and extracts were prepared in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Final concentration of the effectors was 6×10^{-5} M in all cases. Methods were as described in the text. Specific activity is defined as mμ M DAHP produced/mg protein/min.

*Combination of tyrosine, tryptophan and phenylalanine each at the concentration tested individually.

Table 4. Specific activity and inhibition patterns of crude extracts prepared with different concentrations of protamine sulfate

Protamine sulfate concentration (%)	Specific Activity	Effector	Percent inhibition of control activity
0.0	18	TYRO	65
		TRYP	40
		PHE	0
		ARO	85
1.0	23	TYRO	61
		TRYP	42
		PHE	2
		ARO	95
1.5	35	TYRO	63
		TRYP	28
		PHE	12
		ARO	99
2.0	31	TYRO	58
		TRYP	59
		PHE	0
		ARO	94

Extract, protamine sulfate and effectors were prepared in 0.05 M potassium phosphate buffer, pH 7.0, + 0.1 mM $CoCl_2$. Final concentration of effectors was 6×10^{-4} M in all cases. Protamine sulfate treatment and inhibition assays were done as described in Materials and Methods.

Table 5. Specific activity and inhibition patterns of extracts prepared in the presence and absence of NaF

NaF Concentration (M)	Specific Activity	Effectors	Percent Inhibition
0.00	50	TYRO	85
		TRYP	29
		PHE	0
		ARO	95
0.031	45	TYRO	86
		TRYP	7
		PHE	0
		ARO	96
0.313	35	TYRO	88
		TRYP	15
		PHE	0
		ARO	97

Extracts and effectors were prepared in 0.05 M potassium phosphate buffer, pH 7.0, + 0.1 mM $CoCl_2$, with NaF added to the concentrations noted in the table. Final concentration of the effectors was 6×10^{-4} M in all cases. Inhibitions were done as described in Materials and Methods.

thetase was determined in the absence of NaF. Therefore, NaF was excluded from all the buffers used.

It was convenient to store extracts at -70 C and therefore it was necessary to determine the activity of the enzyme over prolonged periods of storage. The activity and inhibition patterns of DAHP synthetase in these extracts did not change significantly at this temperature over the time period shown in Figure 3.

After these conditions of enzyme preparation and storage were determined, the possibility of isozymic forms of DAHP synthetase was investigated. Physical separation of isozyme activities was attempted by ammonium sulfate fractionation. Each fraction was examined for activity and inhibition by the aromatic amino acids (Table 6). Fractions 45 to 55% and 55 to 65% saturation appeared to contain the major portion of DAHP synthetase activity. Inhibition of enzyme activity by tyrosine was significant in all fractions, while tryptophan inhibited the activity found mostly in fractions recovered at 35 to 45% and 45 to 55% saturation. Phenylalanine inhibited the activity found in fraction at 45 to 55% saturation only. This variability in inhibition patterns of the DAHP synthetase activity found in the various ammonium sulfate fractions suggested the presence of isozymes.

Separation of the two activities of DAHP synthetase from crude extracts of Vibrio MB22 was first achieved by chromatography on a DE-32 cellulose column, using a 0.0 to 0.5 M KCl linear gradient (Figure 4). The two activities were tested for inhibition by the aromatic amino acids and the results are given in Table 7. From this data, it was concluded that the first DAHP synthetase activity eluted off the column was a single molecular form inhibited by tyrosine. The latter activity eluted

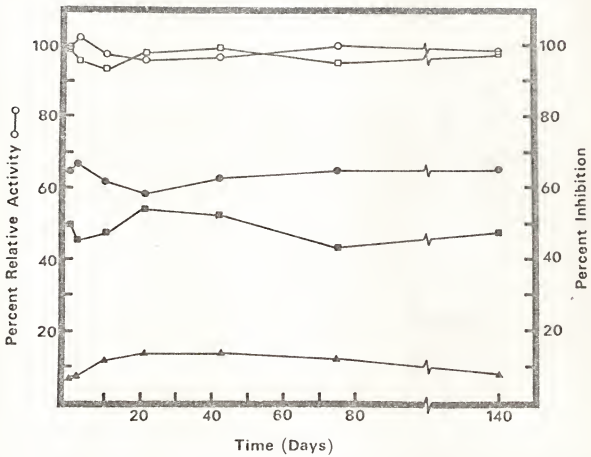

Figure 3. Effect of storage at -70 C on activity and sensitivity to inhibition of DAHP synthetase. Extract was prepared according to the method described in the text, with the exception of the protamine sulfate treatment. Aliquots were frozen at -70 C in the Revco freezer. At the designated times an aliquot was thawed and assayed for DAHP synthetase activity and sensitivity to inhibition by the aromatic amino acids, as described in the text. Symbols for aromatic amino acid inhibition: Tyrosine (●), Tryptophan (■), Phenylalanine (▲), Aro (□).

Table 6. Ammonium sulfate fractionation of crude extract from *Vibrio* MB22

Percent $(NH_4)_2SO_4$ Added	Percent Total Activity	Effector	Percent Inhibition
35-45	2	TYRO	65
		TRYP	55
		PHE	0
		ARO	97
45-55	35	TYRO	60
		TRYP	30
		PHE	21
		ARO	100
55-65	59	TYRO	68
		TRYP	18
		PHE	0
		ARO	96
65-75	8	TYRO	80
		TRYP	0
		PHE	0
		ARO	95

Extract and effectors were prepared in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Final concentration of the effectors was 6×10^{-4} M in all cases. Ammonium sulfate fractionation was done as described in Materials and Methods.

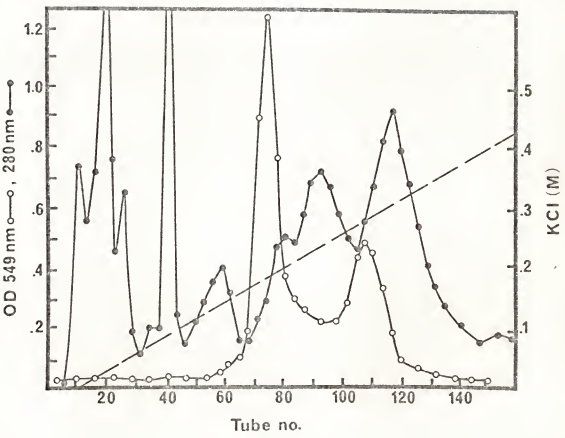

Figure 4. Chromatography of crude extract on DE-32 cellulose (1.5 x 25 cm).
About 200 mg of protein (crude extract) was put onto a DE-32 cellulose column (1.5 x 25 cm). The DAHP synthetase activities were eluted with a 0.0 M to 0.5 M KCl linear gradient formed by mixing 200 ml volumes of each molarity salt made in 0.05 M potassium phosphate buffer + 0.1 mM $CoCl_2$, pH 7.0 buffer. The flow rate was 20 ml/hr and 2.1 ml effluent was collected per tube. Recovery of DAHP synthetase activity was approximately 90%. DAHP synthetase activity was assayed as described in the text.

Table 7. Percent inhibition of DAHP synthetase activities separated on DE-32 cellulose (1.5 x 25 cm)

Effector Concentration (M)	Effector	Peak I (tubes 72, 73)	Peak II (tubes 106, 107)
6×10^{-5}	TYRO	57	20
	TRYP	0	75
	PHE	0	32
	ARO	62	80
6×10^{-4}	TYRO	97	21
	TRYP	9	81
	PHE	17	15
	ARO	100	88

The indicated tubes were combined and DAHP synthetase activity assayed in the presence of the effectors at the final concentrations noted.

off the column was less clearly defined. It was mostly inhibited by tryptophan, although with significant inhibition by tyrosine and phenylalanine even at the lower concentration of the effectors. It was assumed that the second area of activity was in reality a mixture of isozymes.

Crude extract was chromatographed next on a larger (2.5 x 45 cm) DE-32 column and the DAHP synthetase activities was eluted with a 0.1 to 0.4 M KCl linear gradient. Three activities were separated with this procedure (Figure 5). The peak tube of each activity was tested with the three effectors, individually and in combination (Table 8). The first activity eluted off the column was entirely inhibited by tyrosine, while the third activity eluted off the column showed mostly inhibition by tryptophan. The second activity off the column appeared to be a mixture of isozymes, in that it was inhibited by all three effectors, although mostly by phenylalanine.

Hydroxylapatite chromatography was tried next in an attempt to get better separation of the DAHP synthetase activities, especially the one inhibited primarily by phenylalanine. Three activities again were separated using this support and eluting with a 0.05 to 0.3 M potassium phosphate linear gradient (Figure 6). The peak tube of each activity was tested with the aromatic amino acids (Table 9). The results suggest that there were three activities, each primarily responsive to one effector. The small shoulders of activity on the trailing edge of the tyrosine sensitive activity appeared to be a mixture of isozymes with a phenylalanine sensitive isozyme predominating. From this point on the separated DAHP isozymes will be denoted by their primary aromatic amino acid inhibitors, as DAHP synthetase (tyro), DAHP synthetase (tryp) and DAHP synthetase (phe).

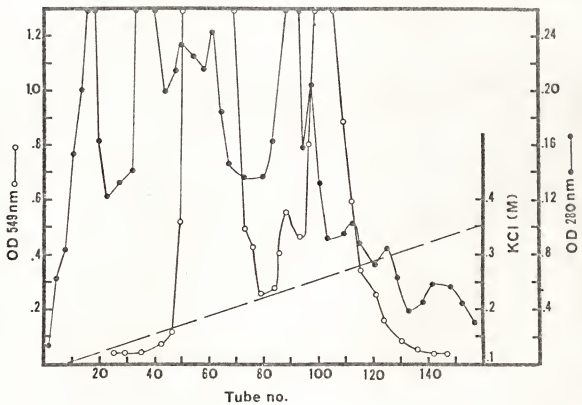

Figure 5. Chromatography of crude extract on DE-32 cellulose (2.5 x 45 cm).
About 1.2 gram of protein (crude extract) was put onto a DE-32 cellulose column (2.5 x 45 cm). The DAHP synthetase activities were eluted with a 0.1 M to 0.4 M KCl linear gradient formed by mixing 500 ml volumes of each molarity salt made in 0.05 M potassium phosphate buffer + 0.1 mM $CoCl_2$, pH 7.0 buffer. The flow rate was 35 ml/hr and 5.2 ml of effluent per tube was collected. Recovery of DAHP synthetase activity was about 95%. DAHP synthetase activity was essayed as described in the text.

Table 8. End product inhibition of DAHP synthetase activities separated by DE-32 cellulose (2.5 x 45 cm)

Tube #	Effector	Percent Inhibition
55	TYRO	98
	TRYP	0
	PHE	0
	ARO	98
88	TYRO	72
	TRYP	33
	PHE	87
	ARO	98
100	TYRO	12
	TRYP	71
	PHE	1
	ARO	80

The tube numbers relate to Figure 5. Final concentration of effectors was 6×10^{-4} M in all cases.

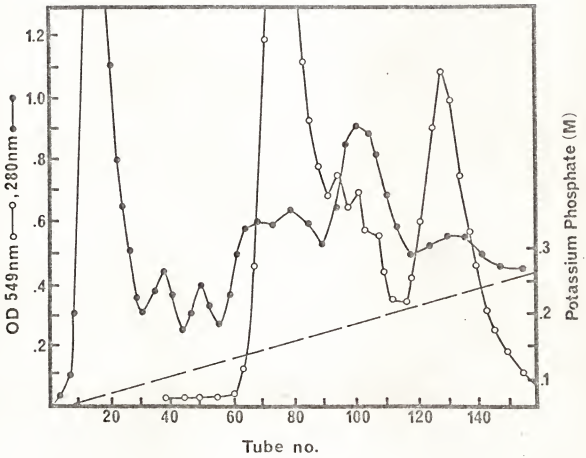

Figure 6. Chromatography of crude extract on hydroxylapatite. About 240 mg of protein (crude extract) was put onto a hydroxylapatite column (1.5 x 25 cm) and eluted with a 0.05 M to 0.3 M potassium phosphate linear gradient. The pH was 6.9, the buffer contained 0.1 mM $CoCl_2$ and the total volume of buffer used was 400 ml. The column was run with a flow rate of 20 ml/hr and 2.1 ml effluent per tube was collected. Recovery of DAHP synthetase activity was about 90%. DAHP synthetase activity was assayed as described in the text.

Table 9. End product inhibition of DAHP synthetase activities separated by hydroxylapatite

Tube #	Effector	Percent Inhibition
73	TYRO	99
	TRYP	8
	PHE	8
	ARO	99
94	TYRO	71
	TRYP	37
	PHE	94
	ARO	98
106	TYRO	34
	TRYP	43
	PHE	94
	ARO	100
127	TYRO	0
	TRYP	76
	PHE	2
	ARO	76

The tube numbers relate to Figure 6. Final concentration of the effectors was 6×10^{-4} M in all cases.

To further separate and purify the isozymes, fractions containing the three different activities eluted from the large DE-32 cellulose column were separately pooled as follows: DAHP synthetase (tyro), tubes 59 to 72; DAHP synthetase (phe), tubes 84 to 92; DAHP synthetase (tryp), tubes 95 to 115. Each activity was concentrated by dialysis in polyethylene glycol at 4 C and rechromatographed on hydroxylapatite. Elution, in each case, showed the presence of a single symmetrical peak of DAHP synthetase activity (Figures 7, 8, 9). These activities eluted in the same position as the crude extract activities on hydroxylapatite. The peak tube from each of these three activities was examined for inhibition by the aromatic amino acids (Table 10). The phenylalanine sensitive activity still showed some inhibition by tyrosine and tryptophan at effector concentration of 6×10^{-4} M. However, this would appear to be mostly non-specific inhibition at this high concentration of effector, as will be seen later. DAHP synthetase activity, pooled from the central portion of each peak of activity, was concentrated and used in other studies. The level of purification and the methods used are summarized in Tables 11, 12 and 13. The isozymes inhibited by tyrosine, tryptophan and phenylalanine, respectively, were purified 71-, 72- and 84-fold.

Previous work (114) using crude extracts indicated that DAHP synthetase activity is stimulated by divalent cations. For this reason, the effect of EDTA and divalent cations on DAHP synthetase activity at various steps in purification was investigated. It was noted that in crude extract the DAHP synthetase activity was reduced to 20% of its control activity in the presence of 0.1 mM EDTA. Table 14 shows that Co^{++} is the most effective in reactivation of this enzymatic activity

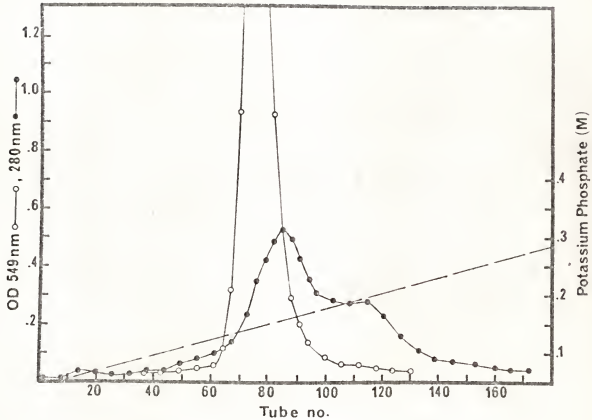

Figure 7. Chromatography of partially purified, tyrosine sensitive DAHP synthetase on hydroxylapatite. The effluent collected in tubes from under the first peak of DAHP synthetase activity from a DE-32 column (Figure 5) was pooled. The protein was concentrated by dialysis in 25% polyethylene glycol at 4 C. This material (about 60 mg of protein) was put onto a hydroxylapatite column (1.5 x 25 cm) and eluted with a 0.05 M to 0.3 M potassium phosphate linear gradient. The total volume of buffer used was 400 ml, the pH was 6.9 and the buffer contained 0.1 mM $CoCl_2$. The flow rate of the column was adjusted to 20 ml/hr and 2.1 ml of effluent per tube was collected. Recovery of DAHP synthetase activity was approximately 90%. DAHP synthetase activity was measured as described in the text.

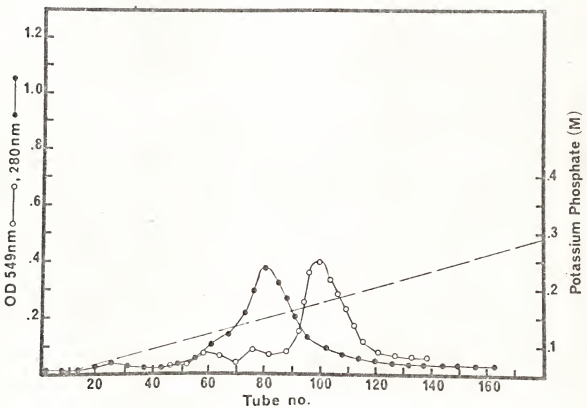

Figure 8. Chromatography of partially purified, phenylalanine sensitive DAHP synthetase on hydroxylapatite. The effluent collected in tubes from under the second peak of DAHP synthetase activity from a DE-32 column (Figure 5) was pooled. The protein was concentrated by dialysis in 25% polyethylene glycol at 4 C. This material (about 25 mg of protein) was put on a hydroxylapatite column (1.5 x 25 cm) and eluted with a 0.05 M to 0.3 M potassium phosphate linear gradient. The total volume of buffer used was 400 ml, the pH was 6.9 and the buffer contained 0.1 mM $CoCl_2$. The flow rate of the column was adjusted to 20 ml/hr and 2.1 ml of effluent per tube was collected. Recovery of DAHP synthetase activity was approximately 60%. DAHP synthetase activity was measured as described in the text.

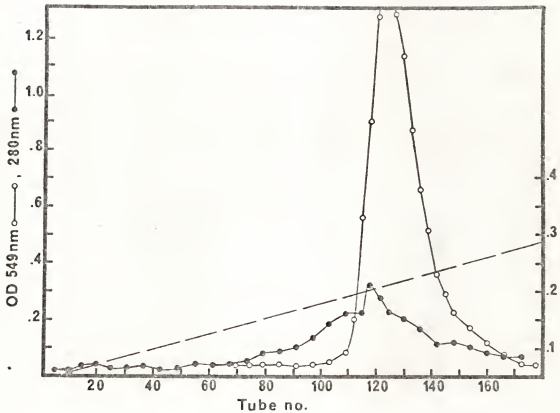

Figure 9. Chromatography of partially purified, tryptophan sensitive DAHP synthetase on hydroxylapatite. The effluent collected in tubes from under the third peak of DAHP synthetase activity from a DE-32 column (Figure 5) was pooled. The protein was concentrated by dialysis in 25% polyethylene glycol at 4 C. This material (about 35 ml of protein) was put on a hydroxylapatite column (1.5 x 25 cm) and eluted with a 0.05 M to 0.3 M potassium phosphate linear gradient. The total volume of buffer used was 400 ml, the pH was 6.9 and the buffer contained 0.1 mM $CoCl_2$. The flow rate of the column was adjusted to 20 ml/hr and 2.1 ml of effluent per tube was collected. Recovery of DAHP synthetase activity was approximately 90%. DAHP synthetase activity was measured as described in the text.

Table 10. End product inhibition of partially purified isozymes rechromatographed on hydroxylapatite

DAHP Isozyme	Tube #	Effector	Percent Inhibition
Tyro	76	TYRO	99
		TRYP	0
		PHE	0
		ARO	99
Phe	98	TYRO	59
		TRYP	31
		PHE	95
		ARO	100
Tryp	124	TYRO	5
		TRYP	75
		PHE	1
		ARO	80

Inhibitions assays were done on the peak tubes indicated above, from column separations illustrated in Figures 7, 8 and 9, respectively. All methods are as described in the text.

Table 11. Purification of the DAHP synthetase (tryo) isozyme

	Fraction	Vol (ml)	Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purification
Step 1	Cell extract	70	1,750	19,200	11.0 (6.6)*	1
Step 2	Protamine sulfate treatment	81	1,330	18,500	14.0 (8.4)*	1.2
Step 3	DE-32 Cellulose chromatography	7	99	6,370	64	10
Step 4	Hydroxylapatite chromatography	5	10	5,500	455	71

*DAHP synthetase (tyro) is approximately 60% of the total crude extract activity. One unit of enzyme activity is defined as the amount of enzyme that catalyzes the formation of 1 mμmole of DAHP per minute.

Table 12. Purification of the DAHP synthetase (tryp) isozyme

	Fraction	Vol (ml)	Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purification
Step 1	Cell extract	148	3,850	77,000	20 (6)*	1
Step 2	Protamine sulfate treatment	162	2,070	75,500	36 (11)*	1.9
Step 3	DE-32 Cellulose chromatography	5	122	7,450	61	10
Step 4	Hydroxylapatite chromatography	12	31	2,500	82	12

*DAHP synthetase (tryp) activity is approximately 30% of the crude extract activity.

Table 13. Purification of the DAHP synthetase (phe) isozyme

	Fraction	Vol (ml)	Protein (mg)	Total Activity (units)	Specific Activity (units/mg)	Purification
Step 1	Cell extract	179	6,170	123,400	20 (2)*	1.0
Step 2	Protamine sulfate treatment	193	3,210	106,000	33 (3.3)*	1.7
Step 3	DE-32 Cellulose chromatography	20	108	6,780	63	32
Step 4	Hydroxylapatite chromatography	8	32	5,330	167	84

*DAHP synthetase (phe) activity is approximately 10% of the crude extract activity.

Table 14. Effect of divalent cations on DAHP synthetase activity in crude extract in the presence and absence of EDTA

EDTA	Divalent Cation	% Relative Activity
-	-	100
+	-	40
+	$CoCl_2$	237
+	$ZnCl_2$	78
+	$SnCl_2 \cdot 2H_2O$	46
+	$MnCl_2 \cdot 4H_2O$	89
+	$CaCl_2$	37
+	$NiCl_2 \cdot 6H_2O$	71
-	$CoCl_2$	274
-	$ZnCl_2$	86
-	$SnCl_2 \cdot 2H_2O$	121
-	$MnCl_2 \cdot 4H_2O$	132
-	$CaCl_2$	132
-	$NiCl_2 \cdot 6H_2O$	160

EDTA and salts were prepared in 0.05 M potassium phosphate buffer, pH 7.0. EDTA and salts, when present, were at a final concentration of 0.1 mM. Methods were as described in the text.

in the presence of 0.1 mM EDTA, while Mn^{++}, Zn^{++}, Ni^{++}, and Sn^{++} have a lesser effect. In the absence of EDTA, only Zn^{++} did not activate the enzyme. As the concentration of Co^{++} was increased (Figure 10) the DAHP synthetase activity in crude extract increased to a maximum of 5-fold at 1 mM Co^{++}. Inhibition patterns of this enzyme in crude extract at various levels of Co^{++} indicated an increase in tyrosine inhibition and decrease of tryptophan and phenylalanine inhibition of DAHP synthetase activity. Tyrosine inhibited the DAHP synthetase activity a maximum of 80% in the presence of 1 mM Co^{++}. The enzymatic activity was inhibited only 36% when EDTA was present at 0.1 mM. Other divalent cations did not appear to alter the inhibition patterns of DAHP synthetase activity in crude extract as drastically as did $CoCl_2$ at 0.1 mM (Table 15).

DAHP synthetase isozymes isolated after DE-32 cellulose chromatography were examined for reactivation of enzyme activity by divalent cations, when the isozymes were in the presence of EDTA. The isozymes were first incubated with 0.1 mM EDTA at 4 C for 10 minutes, then the divalent cation to be tested was added to a final concentration of 0.1 mM and again incubated for 10 minutes at 4 C. The mixtures were then assayed for enzyme activity. DAHP synthetase (tyro) activity is regained (Table 16) most significantly in the presence of Co^{++} followed by Mn^{++}, Ni^{++}, Zn^{++}, Sn^{++} and Ca^{++}, in that order. Since Co^{++} and Mn^{++} were most effective, both cations were tested at various concentrations (Figures 11 and 12). Inhibition of enzyme activity by tyrosine was also examined at these cation concentrations. In Figure 11, Co^{++} maximally activates DAHP synthetase (tyro) at a concentration of 10^{-3} to 10^{-2} M. Inhibition of the enzyme activity by 3×10^{-5} M tyrosine

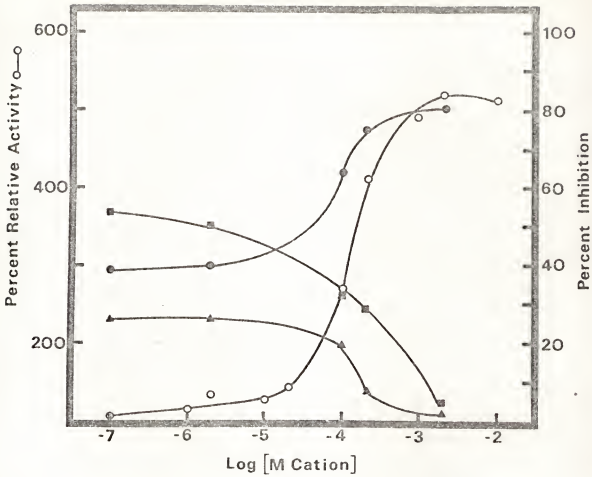

Figure 10. Activity and inhibition patterns of DAHP synthetase in crude extract at varied concentrations of Co^{++} in the presence of EDTA. Extract was prepared in 0.05 M potassium phosphate buffer without protamine sulfate treatment. $CoCl_2$, aromatic amino acids and EDTA were also prepared in the same buffer. EDTA was present at a final concentration of 0.1 mM. Amino acids were present at 6×10^{-4} M final concentration in all cases. Methods were as described in the text.
Symbols for aromatic amino acid inhibition: Tyrosine (●), Tryptophan (■), Phenylalanine (▲).

Table 15. Effect of EDTA, divalent cations on inhibition of DAHP synthetase activity in crude extract

EDTA	Cation	Effectors	% Inhibition
--	--	TYRO	50
		TRYP	41
		PHE	17
		ARO	90
+	--	TYRO	26
		TRYP	43
		PHE	21
		ARO	88
+	$CoCl_2$	TYRO	75
		TRYP	22
		PHE	17
		ARO	95
+	$ZnCl_2$	TYRO	31
		TRYP	57
		PHE	10
		ARO	98
+	$MnCl_2 \cdot 4H_2O$	TYRO	25
		TRYP	43
		PHE	7
		ARO	88
+	$NiCl_2 \cdot 6H_2O$	TYRO	43
		TRYP	51
		PHE	21
		ARO	87
+	CaCl	TYRO	35
		TRYP	48
		PHE	14
		ARO	92
+	$SnCl_2 \cdot 2H_2O$	TYRO	42
		TRYP	37
		PHE	16
		ARO	90

EDTA, salts and effectors were prepared in 0.05 M potassium phosphate buffer, pH 7.0. EDTA and salts, when present, were at a final concentration of 0.1 mM. Final concentration of effectors was 6×10^{-4} M in all cases. Methods were as described in the text.

Table 16. Effect of EDTA and divalent cations on the activity and the tyrosine inhibition of DAHP synthetase (tyro) after DE-32 cellulose chromatography

EDTA	Cation	% Relative Activity	Percent Inhibition in the Presence of Tyrosine
-	-	100	17
+	-	18	58
+	$CoCl_2$	116	38
+	$ZnCl_2$	74	39
+	$SnCl_2 \cdot 2H_2O$	30	50
+	$MnCl_2 \cdot 4H_2O$	89	64
+	$CaCl_2$	24	53
+	$NiCl_2 \cdot 6H_2O$	79	54

EDTA, salts and tyrosine were prepared in 0.05 M potassium phosphate buffer, pH 7.0. EDTA and salts, when present, were at a final concentration of 0.1 mM. The final concentration of tyrosine was 3×10^{-5} M in all cases. Methods were as described in the text.

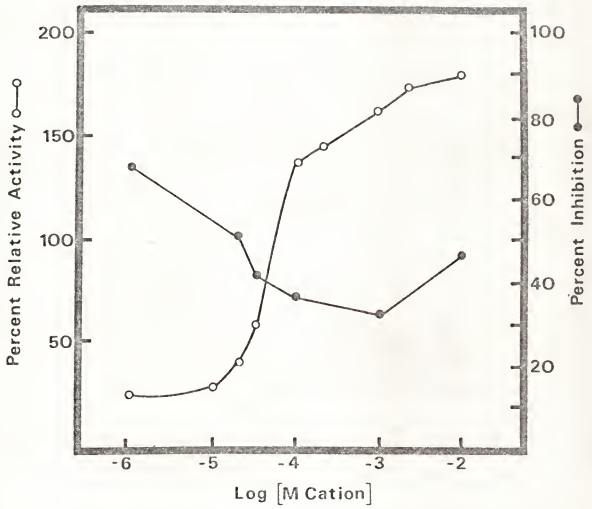

Figure 11. Activity and tyrosine inhibition of DE-32 cellulose chromatographed DAHP synthetase (tyro) at varied concentrations of Co^{++} in the presence of EDTA.
DAHP synthetase (tyro) pooled and concentrated after elution from DE-32 cellulose (Figure 5) was used. $CoCl_2$, tyrosine and EDTA were prepared in 0.05 M potassium phosphate buffer. Tyrosine was present at a final concentration of 3×10^{-5} M. EDTA was present at a final concentration of 0.1 mM. Methods were as described in the text.

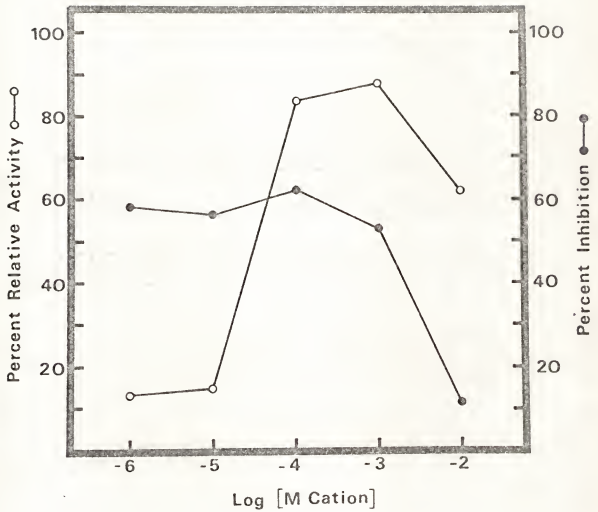

Figure 12. Activity and tyrosine inhibition of DE-32 cellulose chromatographed DAHP synthetase (tyro) at varied concentrations of Mn^{++} in the presence of EDTA.
DAHP synthetase (tyro) pooled and concentrated after elution from DE-32 cellulose (Figure 5) was used. $MnCl_2 \cdot 4H_2O$, tyrosine and EDTA were prepared in 0.05^2M potassium phosphate buffer. EDTA was present at a final concentration of 0.1 mM. Tyrosine was present at a final concentration of 3×10^{-5} M. Methods were as described in the text.

is least at a Co^{++} concentration of 10^{-3} M. Figure 12 shows that Mn^{++} maximally activates the DAHP synthetase (tyro) at a concentration of 10^{-3} M, while the sensitivity of the enzyme to tyrosine inhibition is least at concentrations of Mn^{++} above 10^{-3} M.

DAHP synthetase (tryp) is reactivated to the greatest extent by Mn^{++} while Co^{++}, Ni^{++} and Zn^{++}, in that order, are less effective (Table 17). Cobalt reactivation is maximal at a concentration of 10^{-4} M while the sensitivity of the enzyme to inhibition by tryptophan increases gradually with an increase in Co^{++} concentration (Figure 13). The maximum regain of catalytic activity in the presence of Mn^{++} occurs at a concentration of 10^{-3} M, as does the maximum sensitivity of the enzyme to inhibition by tryptophan (Figure 14). It should be noted, however, that DAHP synthetase (tryp) is less sensitive to inactivation by EDTA than DAHP synthetase (tyro).

DAHP synthetase (Phe) is inactivated more by EDTA (Table 18) than DAHP synthetase (tryp) and is reactivated most effectively by Mn^{++} followed by Sn^{++}, Co^{++}, Zn^{++}, Ca^{++} and Ni^{++}, in that order. This isozyme is maximally activated and is most sensitive to inhibition by phenylalanine at a concentration of 10^{-4} M Co^{++} (Figure 15). Tin activates most effectively at a concentration of 10^{-3} M, while the sensitivity of the enzyme to inhibition with phenylalanine increases as the concentration of Sn^{++} increases (Figure 16). Manganese activates this catalytic activity approximately twice the control activity between concentrations of 10^{-4} and 10^{-3} M Mn^{++}, while sensitivity to inhibition by phenylalanine increases with Mn^{++} concentration (Figure 17).

Monovalent cations tested in the presence of $CoCl_2$ and absence of EDTA (Table 19) showed little or no activation or inactivation in some cases. The same results were obtained when 0.1 mM EDTA was present.

Table 17. Effect of EDTA and divalent cations on the activity and the tryptophan inhibition of DAHP synthetase (tryp) after DE-32 cellulose chromatography

EDTA	Cation	% Relative Activity	Percent Inhibition in the Presence of Tryptophan
-	-	100	39
+	-	51	34
+	$CoCl_2$	87	47
+	$ZnCl_2$	60	48
+	$SnCl_2 \cdot 2H_2O$	46	31
+	$MnCl_2 \cdot 4H_2O$	90	37
+	$CaCl_2$	50	36
+	$NiCl_2 \cdot 6H_2O$	67	38

EDTA, salts and tryptophan were prepared in 0.05 M potassium phosphate buffer, pH 7.0. EDTA and salts, when present, were at a final concentration of 0.1 mM. The final concentration of tryptophan was 6×10^{-5} M in all cases. Methods were as described in the text.

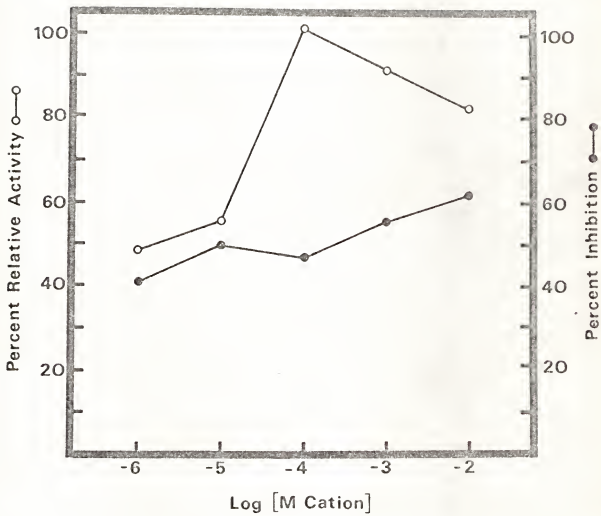

Figure 13. Activity and tryptophan inhibition of DE-32 cellulose chromatographed DAHP synthetase (tryp) at varied concentrations of Co^{++} in the presence of EDTA.
DAHP synthetase (tryp) pooled and concentrated after elution from DE-32 cellulose (Figure 5) was used. $CoCl_2$, tryptophan and EDTA were prepared in 0.05 M potassium phosphate buffer. EDTA was present at 0.1 mM final concentration. Tryptophan was present at a final concentration of 6×10^{-5} M. Methods were as described in the text.

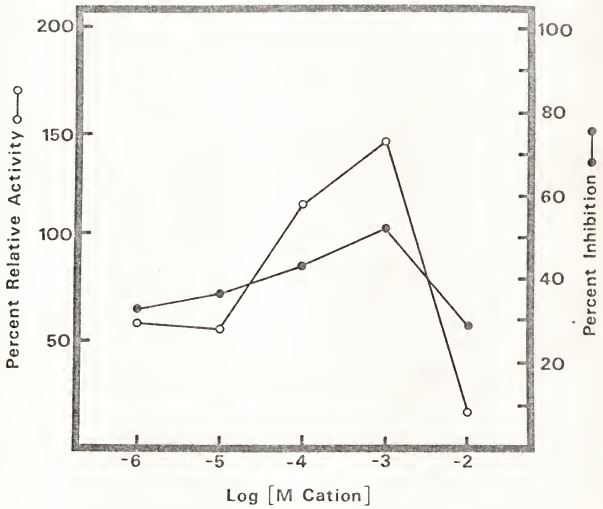

Figure 14. Activity and tryptophan inhibition of DE-32 cellulose chromatographed DAHP synthetase (tryp) at varied concentrations of Mn^{++} in the presence of EDTA.
DAHP synthetase (tryp) pooled and concentrated after elution from DE-32 cellulose (Figure 5) was used. $MnCl_2 \cdot 4H_2O$, tryptophan and EDTA were prepared in 0.05 M potassium phosphate buffer. EDTA was present at 0.1 mM final concentration. Tryptophan was present at a final concentration of 6×10^{-5} M. Methods were as described in the text.

Table 18. Effect of EDTA and divalent cations on the activity and the phenylalanine inhibition of DAHP synthetase (phe) after DE-32 cellulose chromatography

EDTA	Cation	% Relative Activity	Percent Inhibition in the Presence of Phenylalanine
-	-	100	76
+	-	36	40
+	$CoCl_2$	79	71
+	$ZnCl_2$	96	46
+	$SnCl_2 \cdot 2H_2O$	156	41
+	$MnCl_2 \cdot 4H_2O$	159	57
+	$CaCl_2$	99	42
+	$NiCl_2 \cdot 6H_2O$	28	44

EDTA, salts and phenylalanine were prepared in 0.05 M potassium phosphate buffer, pH 7.0. EDTA and salts, when present, were at a final concentration of 0.1 mM. The final concentration of phenylalanine was 3×10^{-6} M in all cases. Methods were as described in the text.

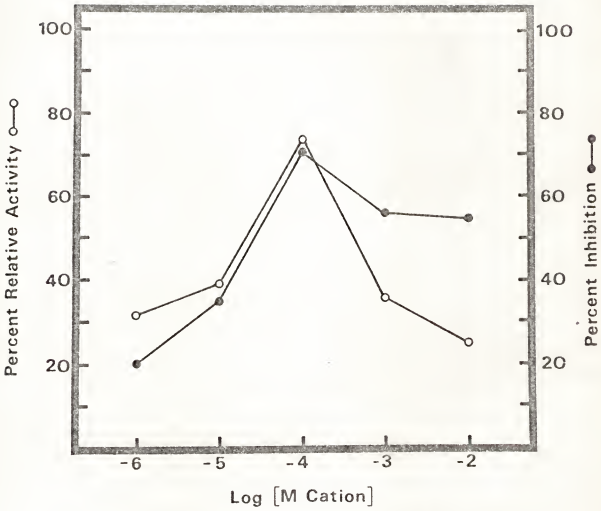

Figure 15. Activity and phenylalanine inhibition of DE-32 cellulose chromatographed DAHP synthetase (phe) at varied concentrations of Co^{++} in the presence of EDTA. DAHP synthetase (phe) pooled and concentrated after elution from DE-32 cellulose (Figure 5) was used. $CoCl_2$, phenylalanine and EDTA were prepared in 0.05 M potassium phosphate buffer. EDTA was present at 0.1 mM final concentration. Phenylalanine was present at a final concentration of 3×10^{-6} M. Methods were as described in the text.

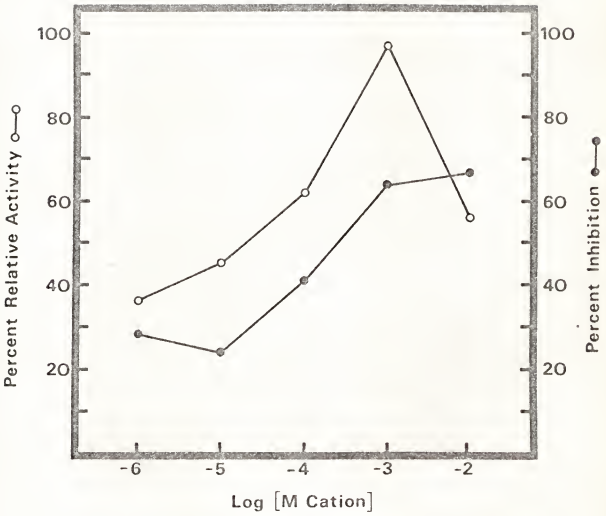

Figure 16. Activity and phenylalanine inhibition of DE-32 cellulose chromatographed DAHP synthetase (phe) at varied concentrations of Sn^{++} in the presence of EDTA.
DAHP synthetase (phe) pooled and concentrated after elution from DE-32 cellulose (Figure 5) was used. $SnCl_2 \cdot 2\ H_2O$, phenylalanine and EDTA were prepared in 0.05 M potassium phosphate buffer. EDTA was present at 0.1 mM final concentration. Phenylalanine was present at a final concentration of 3×10^{-6} M. Methods were as described in the text.

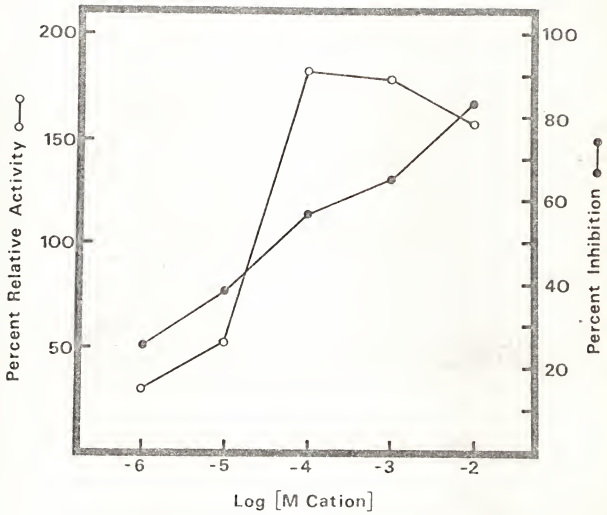

Figure 17. Activity and phenylalanine inhibition of DE-32 cellulose chromatographed DAHP synthetase (phe) at varied concentrations of Mn^{++} in the presence of EDTA.
DAHP synthetase (phe) pooled and concentrated after elution from DE-32 cellulose (Figure 5) was used. $MnCl_2 \cdot 4\ H_2O$, phenylalanine and EDTA were prepared in 0.05 M potassium phosphate buffer. EDTA was present at a 0.1 mM final concentration. Phenylalanine was present at a final concentration of 3×10^{-6} M. Methods were as described in the text.

Table 19. Effect of monovalent cations on the activity of the isozymes after DE-32 cellulose chromatography

Salts	Percent relative activity of isozymes		
	Tyro	Tryp	Phe
--	100	100	100
NaCl	129	81	75
KCl	98	76	78
LiCl	107	72	73
NH_4Cl	91	80	65
NaF	127	86	68
KF	115	101	81

Salts were prepared in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Salts, when present, were at a final concentration of 0.1 M. Methods were as described in the text.

All of the isozymes after rechromatography on hydroxylapatite were no longer affected by EDTA or by divalent cations (Table 20). Monovalent cations in the presence of $CoCl_2$ and absence of EDTA had little or no effect on enzyme activity (Table 21).

An attempt was made to see if the proteins separated from the isozyme by hydroxylapatite chromatography could be added back to the enzyme in order to reestablish the divalent cation activation characteristic. DAHP synthetase (tyro) eluted from a DE-32 cellulose column was rechromatographed on hydroxylapatite (Figure 18). The DAHP synthetase activity (tubes 55 to 85) and the protein fractions (I = tubes 1 to 54; II = tubes 86 to 146, III = 60 ml of 0.5 M potassium phosphate effluent after tube 146) were pooled separately and concentrated by dialysis with polyethylene glycol. It was found that this DAHP synthetase activity was unstable at 27 C (Table 22), and EDTA and divalent cations were still unable to affect the enzyme. The protein fractions isolated from the column and bovine serum albumin (BSA) were tested to see if they could stabilize this isozyme. These proteins were added to the isozyme as noted in Table 22 and preincubated for 30 minutes at 27 C and then assayed for activity. Without added protein, activity was reduced to 19% in 30 minutes. Protein fractions I and II were capable of stabilizing the isozyme more effectively than much higher concentrations of BSA. Supernatants of boiled protein fractions I and II did stabilize, but less effectively than before boiling.

DAHP synthetase in crude extract as well as isolated enzymes were examined for stability in the presence of their most effective divalent cation activator, either Mn^{++} or Co^{++}, EDTA and PEP. The compounds were added individually or in combination to the enzyme as noted in the

Table 20. Effect of EDTA and divalent cations on the activity of the DAHP synthetase isozymes after hydroxylapatite chromatography*

EDTA	Cation	Percent relative activity of isozymes		
		Tyro	Tryp	Phe
--	--	100	100	100
+	--	103	104	97
+	$CoCl_2$	105	107	102
+	$ZnCl_2$	86	98	95
+	$SnCl_2 \cdot 2H_2O$	98	94	104
+	$MnCl_2 \cdot 4H_2O$	108	97	103
+	$CaCl_2$	90	100	104
+	$NiCl_2 \cdot 6H_2O$	99	91	94

EDTA and salts were prepared in 0.05 M potassium phosphate buffer pH 7.0. EDTA and salts, when present, were at a final concentration of 0.1 mM. Methods were as described in the text.

*The isozymes were first separated on DE-32 cellulose and each individual isozyme activity was rechromatographed separately on hydroxylapatite.

Table 21. Effect of monovalent cations on the activity of the DAHP synthetase isozymes after hydroxylapatite chromatography*

Cation	Percent relative activity of isozymes		
	Tyro	Tryp	Phe
--	100	100	100
NaCl	99	98	87
KCl	94	96	77
LiCl	97	93	90
NH_4Cl	97	95	92
NaF	119	99	96
KF	111	101	102

Salts were prepared in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Salts, when present, were at a concentration of 0.1 M. Methods were as described in the text.

*The isozymes were first separated on DE-32 cellulose and each individual isozyme activity was rechromatographed separately on hydroxylapatite.

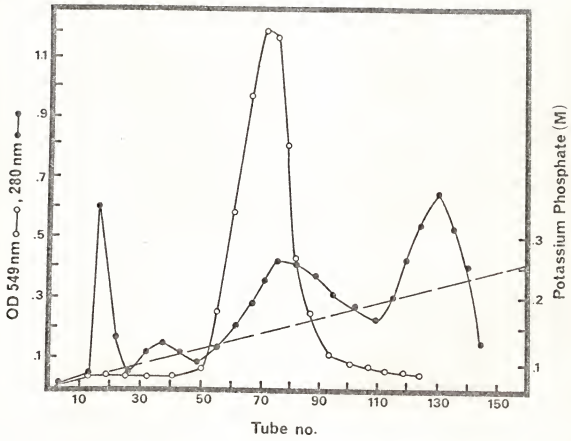

Figure 18. Chromatography of partially purified, tyrosine sensitive DAHP synthetase on hydroxylapatite. Methods for chromatography were as described in Figure 7. DAHP synthetase (tyro) activity, tubes 55-85, was pooled, as well as protein fractions before and after the peak of activity, I = tubes 1-54, II = tubes 86-145. Fraction III was obtained by washing the column with 0.5 M KCl in buffer and collecting 60 ml of effluent. All fractions were concentrated in 25% polyethylene glycol at 4 C.

Table 22. Effect of proteins on the stability of the DAHP synthetase (tyro) after rechromatography on hydroxylapatite

Addition	Percent Relative Activity
No Preincubation	100
None	19
Protein Fraction I (0.8 mg/ml)	56
Protein Fraction II (2.7 mg/ml)	80
Protein Fraction III (1.3 mg/ml)	26
Protein Fraction I + I + III (1.3 mg/ml)	80
BSA (3 mg/ml)	48
BSA (6 mg/ml)	68
BSA (12 mg/ml)	73
Protein Fraction I Supernatant	29
Protein Fraction II Supernatant	52
Protein Fraction III Supernatant	7

Protein fractions (I = tube #1 to 55, II = tube #86 to 145 and III = 60 ml of 0.05 M potassium phosphate effluent) from the hydroxylapatite column (Figure 19) were pooled separately and concentrated by dialysis in polyethylene glycol. Bovine serum allumin (BSA) was prepared in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Supernatants of the protein fractions were prepared by boiling these fractions for 5 minutes and removing the precipitate by centrifugation. Enzyme was preincubated with the protein fractions and BSA at the final concentrations noted in the table for 30 minutes at 27 C. Methods were as described in the text.

tables and preincubated for 30 minutes at 27 C. In some cases the divalent cation or PEP was added after this preincubation. Crude extract DAHP synthetase activity was found to be stable for this preincubation period (Table 23). This crude extract activity was also activated nearly threefold by 0.1 mM Co^{++} and 14% by PEP. EDTA was found to inactivate DAHP synthetase in crude extract and the presence of PEP had no effect on this EDTA inactivation.

DAHP synthetase (tyro) eluted from a DE-32 cellulose column was found to be stable under these conditions of incubation (Table 24). This isozyme was activated by Co^{++} and PEP, and inactivated by EDTA at the indicated concentrations. PEP at a concentration of 3 mM protected DAHP synthetase (tyro) from inactivation by 0.1 mM EDTA. Cobalt also activated this isozyme in the presence of PEP. In contrast, the DAHP synthetase (tyro) separated on a DE-32 cellulose column and rechromatographed on hydroxylapatite was found to be unstable under these conditions (Table 24). Its activity was reduced to 17% in 30 minutes, but the catalytic activity was stabilized if 3 mM PEP was present. Cobalt was ineffective with this isozyme both as a stabilizer or an activator. Phosphoenolpyruvate at a concentration of 3 mM was also found to protect the enzyme in the presence of 0.1 mM EDTA.

DAHP synthetase (tryp) eluted from a DE-32 cellulose column was only slightly unstable during this 30 minutes' incubation (Table 25). This isozyme was stabilized by 3 mM PEP and inactivated by EDTA. Phosphoenolpyruvate at a concentration of 3 mM protected this isozyme from EDTA inactivation. DAHP synthetase (tryp) separated on DE-32 cellulose and rechromatographed on hydroxylapatite was only moderately unstable, as shown by a residual activity of 58% after incubation

Table 23. Effect of Co^{++}, EDTA and phosphoenolpyruvate on the stability of DAHP synthetase activity in crude extract

Addition		Percent Relative Activity
Preincubation	Assay	
No preincubation	--	100
--	--	106
Co^{++} (0.1 mM)	--	272
PEP (3 mM)	--	114
EDTA (0.1 mM)	--	22
--	Co^{++} (0.1 mM)	243
--	PEP (3 mM)	111
EDTA (0.1 mM)	Co^{++} (0.1 mM)	165
EDTA (0.1 mM) + PEP (3 mM)	--	28
EDTA (0.1 mM) + PEP (3 mM)	Co^{++} (0.1 mM)	170
PEP (3 mM)	Co^{++} (0.1 mM)	252
Co^{++} (0.1 mM)	PEP (3 mM)	240

$CoCl_2$, EDTA and phosphoenolpyruvate (PEP) were prepared in 0.05 M potassium phosphate buffer, pH 7.0. Enzyme was preincubated for 30 minutes at 27 C in the presence of these various compounds. In some cases $CoCl_2$ or PEP was added just before the assay. In all experiments where PEP was added the concentration of PEP in the reaction mixture was adjusted so that its final concentration would be the same as in the normal assay. Methods were as described in the text.

Table 24. Effect of Co^{++}, EDTA, PEP and BSA on the stability of the DAHP synthetase (tyro) after DE-32 cellulose and hydroxylapatite chromatography

Addition		Percent Relative Activity	
Preincubation	Assay	DE-32	Hydroxylapatite
No preincubation	--	100	100
--	--	105	17
Co^{++} (1 mM)	--	141	37
PEP (3 mM)	--	125	120
EDTA (0.1 mM)	--	5	1
--	Co^{++} (1 mM)	141	24
--	PEP (3 mM)	115	28
EDTA (0.1 mM)	Co^{++} (1 mM)	74	3
EDTA (0.1 mM) + PEP (3 mM)	--	118	110
EDTA (0.1 mM) + PEP (3 mM)	Co^{++} (1 mM)	147	110
PEP (3 mM)	Co^{++} (1 mM)	140	120
Co^{++} (1 mM)	PEP (3 mM)	153	42
BSA (3 mg/ml)	--	--	48

$CoCl_2$, EDTA, PEP and BSA were prepared in 0.05 M potassium phosphate buffer, pH 7.0. Enzyme was preincubated for 30 minutes at 27 C in the presence of these various compounds. In some cases $CoCl_2$ or PEP was added just before the assay. Methods were as described in the text.

Table 25. Effect of Mn^{++}, EDTA, PEP and BSA on the stability of the DAHP synthetase (tryp) after DE-32 cellulose and hydroxylapatite chromatography

Addition		Percent Relative Activity	
Preincubation	Assay	DE-32	Hydroxylapatite
No preincubation	--	100	100
--	--	88	58
Mn^{++} (1 mM)	--	92	64
PEP (3 mM)	--	101	123
EDTA (0.1 mM)	--	33	49
--	Mn^{++} (1 mM)	95	61
--	PEP (3 mM)	96	64
EDTA (0.1 mM)	Mn^{++} (1 mM)	115	58
EDTA (0.1 mM) + PEP (3 mM)	--	89	134
EDTA (0.1 mM) + PEP (3 mM)	Mn^{++} (1 mM)	120	126
PEP (3 mM)	Mn^{++} (1 mM)	110	127
Mn^{++} (1 mM)	PEP (3 mM)	101	66
BSA (3 mg/ml)	--	--	84

$MnCl_2 \cdot 4H_2O$, EDTA, PEP and BSA were prepared in 0.05 M potassium phosphate buffer, pH 7.0. Enzyme was preincubated for 30 minutes at 27 C in the presence of these various compounds. In some cases $MnCl_2 \cdot 4H_2O$ or PEP were added just before the assay. Methods were as described in the text.

(Table 25). Manganese and EDTA at the concentrations tested had little effect on this enzyme. However, 3 mM PEP did stabilize it and even activated it 23%. Bovine serum albumin at a concentration of 3 mg/ml was also effective to some degree in stabilizing this isozyme.

DAHP synthetase (phe) eluted from DE-32 cellulose was stable to incubation at 27 C (Table 26). Manganese at a concentration of 1 mM and 3 mM PEP activated this isozyme. Inactivation of this isozyme by 0.1 mM EDTA was only 27%. However, 3 mM PEP did protect this isozyme from this minimal inhibition by EDTA. This isozyme after being re-chromatographed on hydroxylapatite was partially unstable (Table 26). Manganese and PEP were only slightly effective in stabilizing this activity. However, the presence of both 0.1 mM EDTA and 3 mM PEP were very effective in stabilization of this isozyme. Bovine serum albumin at a concentration of 3 mg/ml only partially stabilized the enzyme activity.

The most purified forms of the isozymes, that is those separated on DE-32 cellulose and rechromatographed on hydroxylapatite, were used for physical characterizations. These isozymes were examined for the effect of pH on catalytic activity and inhibition. The isozymes were prepared in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$, the column buffer. The isozyme was diluted in 0.2 M buffer of the appropriate pH and incubated for 10 minutes at 4 C. This mixture was then assayed by the usual method. The pH's recorded are the actual pH's measured after dilution. DAHP synthetase (tyro) was most active at pH 7.4 while maximum sensitivity of the enzyme to tyrosine inhibition varied between pH 5.6 and 7.4 (Figure 19). This sensitivity fell off sharply below pH 5.6 and above pH 7.4. DAHP synthetase (tryp) was

Table 26. Effect of Mn^{++}, EDTA, PEP and BSA on the stability of the DAHP synthetase (phe) after DE-32 cellulose and hydroxylapatite chromatography

Addition		Percent Relative Activity	
Preincubation	Assay	DE-32	Hydroxylapatite
No preincubation	--	100	100
--	--	101	69
Mn^{++} (1 mM)	--	163	90
PEP (3 mM)	--	121	85
EDTA (0.1 mM)	--	73	89
--	Mn^{++} (0.1 mM)	99	76
--	PEP (3 mM)	102	70
EDTA (0.1 mM)	Mn^{++} (0.1 mM)	220	84
EDTA (0.1 mM) + PEP (3 mM)	--	120	118
EDTA (0.1 mM) + PEP (3 mM)	Mn^{++} (0.1 mM)	112	117
PEP (3 mM)	Mn^{++} (0.1 mM)	120	109
Mn^{++} (0.1 mM)	PEP (3 mM)	137	81
BSA (3 mg/ml)	--	--	80

$MnCl_2 \cdot 4H_2O$, EDTA, PEP and BSA were prepared in 0.05 M potassium phosphate buffer, pH 7.0. Enzyme was preincubated for 30 minutes at 27 C in the presence of these various compounds. In some cases $MnCl_2 \cdot 4H_2O$ or PEP was added just before the assay. Methods were as described in the text.

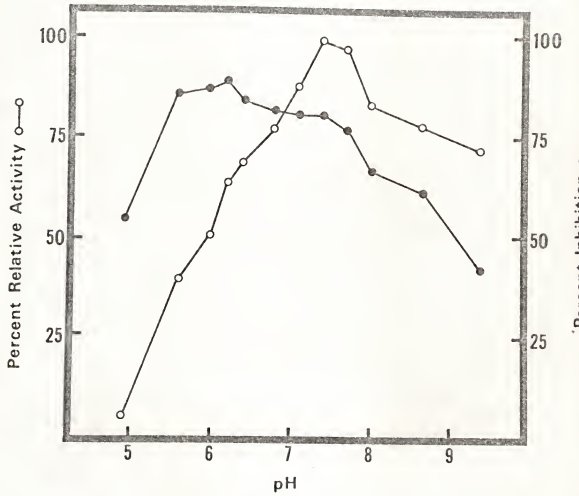

Figure 19. Effect of pH on activity and tyrosine inhibition of DAHP synthetase (tyro). DAHP synthetase (tyro) which was eluted from DE-32 cellulose and rechromatographed on hydroxylapatite was used. This isozyme was present in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Buffers used were: 0.2 M citrate-potassium phosphate buffer, pH 4.8 to 5.6; 0.2 M potassium phosphate buffer, pH 6.0 to 8.0; 0.2 M glycine-sodium hydroxide buffer, pH 9.0 to 10.0. Tyrosine was prepared in each pH buffer tested and present at a final concentration of 6×10^{-5} M. All buffers tested contained 0.1 mM $CoCl_2$. Methods were as described in the text.

also most active at pH 7.4 and most sensitive to tryptophan inhibition between pH 7.2 and 7.6 (Figure 20). DAHP synthetase (phe) had its maximal activity at pH 8.0 and was most sensitive to inhibition by phenylalanine at pH 7.2 (Figure 21).

The apparent molecular weights of the isozymes were determined by gel filtration with Sephadex G-200. From the molecular weight column calibration curve (Figure 22), the crude extract DAHP synthetase activity, as well as DAHP synthetase (tyro),and (tryp) appear to have a molecular weight of approximately 55,000. However, DAHP synthetase (phe) appeared to have a molecular weight of approximately 150,000.

Isozymes separated and partially purified by DE-32 cellulose and hydroxylapatite chromatography were used to study both substrate and inhibition kinetics. In studying substrate kinetics, the initial velocity (v) was determined as a function of the concentration of one substrate, at a fixed and saturating level of the other substrate. The effect of the feedback inhibitors, tyrosine, tryptophan or phenylalanine on the kinetic behavior was also investigated at concentrations resulting in moderate levels of inhibition. The results are presented as double reciprocal (Linweaver-Burke) plots and also Hill plots of log [v/(V-v)] versus log [S].

DAHP synthetase (tyro),with E-4-P as the variable substrate, shows an upward curve of the double reciprocal plot (Figure 23) at higher concentrations of E-4-P, in the presence and absence of tyrosine. V max was obtained from reciprocal plots, showing this type of curve, by extrapolation of the linear portion (at least four points) at high substrate concentrations. Hill plots of this data (Figure 24) gave slopes which are greater than 1, especially in the absence of tyrosine. The

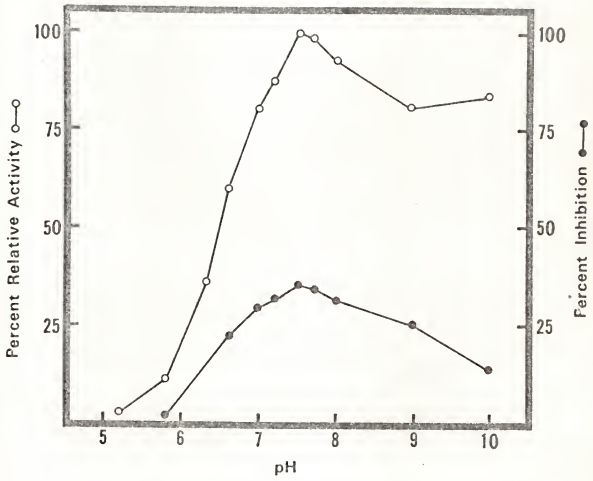

Figure 20. Effect of pH on activity and tryptophan inhibition of DAHP synthetase (tryp). DAHP synthetase (tryp) which was eluted from DE-32 cellulose and rechromatographed on hydroxylapatite was used. This isozyme was present in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Buffers used were: 0.2 M citrate-potassium phosphate buffer, pH 4.8 to 5.6; 0.2 M potassium phosphate buffer, pH 6.0 to 8.0; 0.2 M glycine-sodium hydroxide buffer, pH 9.0 to 10.0. Tryptophan was prepared in each pH buffer tested and present at a final concentration of 3×10^{-5} M. All buffers tested contained 0.1 mM $CoCl_2$. Methods were as described in the text.

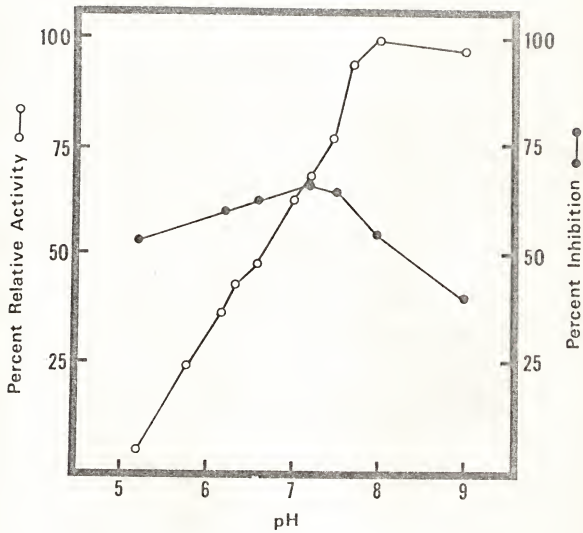

Figure 21. Effect of pH on activity and phenylalanine inhibition of DAHP synthetase (phe). DAHP synthetase (phe) which was eluted from DE-32 cellulose and rechromatographed on hydroxylapatite was used. This isozyme was present in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Buffers used were: 0.2 M citrate-potassium phosphate buffer, pH 4.8 to 5.6; 0.2 M potassium phosphate buffer, pH 6.0 to 8.0; 0.2 M glycine-sodium hydroxide buffer, pH 9.0 to 10.0. Phenylalanine was prepared in each pH buffer tested and was present at a final concentration of 3×10^{-6} M. All buffers tested contained 0.1 mM $CoCl_2$. Methods were as described in the text.

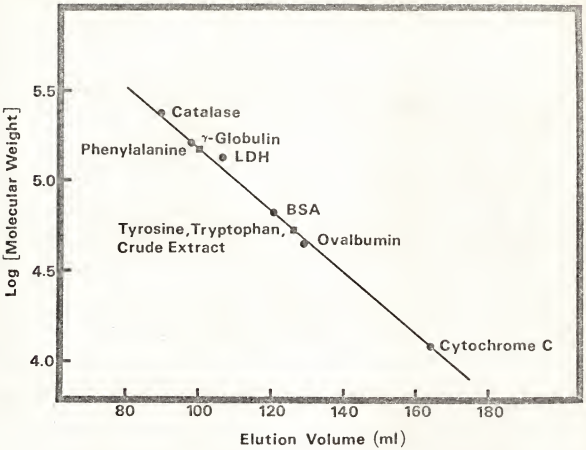

Figure 22. Molecular weight determinations of DAHP synthetase activity in crude extract and isozymes with Sephadex G-200.
Crude extract was prepared as described in the text. Isozymes used were separated on DE-32 cellulose and rechromatographed on hydroxylapatite. The column (2.5 x 40 cm) was equilibrated with 0.05 M potassium phosphate buffer pH 7.0 + 0.1 mM $CoCl_2$ by reverse flow at a rate of 12 ml/hr and 2 ml fractions were collected. All standards, extract and isozymes were prepared in the same buffer. The following amounts of standards were run in 2 ml volumes: 2 mg catalase, bovine liver; 0.3 mg lactate dehydrogenase, rabbit muscle; 3 mg γ-globulin, rabbit; 8 mg bovine serum albumin; 8 mg ovalbumin; 5 mg cytochrome C, horse. Enzyme assays were conducted as described in the text.

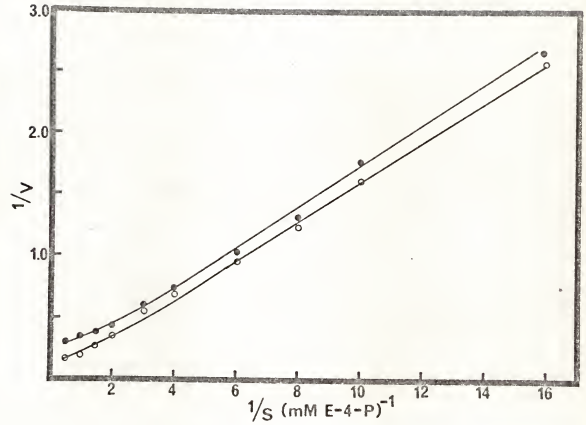

Figure 23. Double reciprocal plot of initial velocity (v) versus erythrose-4-phosphate concentrations for DAHP synthetase (tyro) in the presence and absence of tyrosine. DAHP synthetase (tyro) which was eluted from DE-32 cellulose and rechromatographed on hydroxylapatite was used. The isozyme was present in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Phosphoenolpyruvate was present at a final concentration of 2.8 mM. The reaction mixtures were incubated at 37 C for 10 minutes. The assay was as described in Materials and Methods. Tyrosine was prepared in the same buffer as the isozyme and present at a final concentration of 3×10^{-5} M, which gave approximately 50% inhibition at saturating levels of substrate. Symbols: no tyrosine (o), 3×10^{-5} M tyrosine (●).

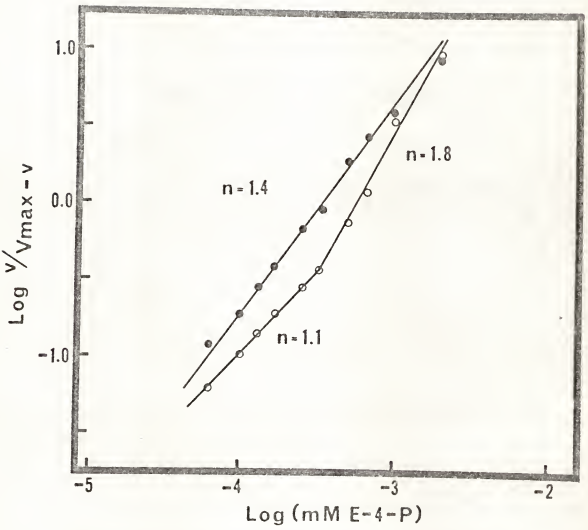

Figure 24. Hill plot of DAHP synthetase (tyro) activity at varied erythrose-4-phosphate concentrations in the presence and absence of tyrosine. The data used in these calculations is the same as that recorded for Figure 23. V max was obtained from extrapolation of the linear portion (four points) at high concentrations of erythrose-4-phosphate in the reciprocal plot, v is the initial velocity. Symbols: no tyrosine (o), 3×10^{-5} M tyrosine (●).

increase in slope at concentrations of E-4-P above 5×10^{-4} M was reproducible. The same isozyme with PEP as the variable substrate also showed an upward curve of the reciprocal plot (Figure 25) at high PEP concentrations, both in the presence and absence of tyrosine. Hill plots (Figure 26) gave slopes of approximately 1.5 for both curves. This data suggested positive cooperativity between substrate binding sites.

DAHP synthetase (tryp) at varied concentrations of E-4-P shows some curvature in the reciprocal plots (Figure 27). However, the slopes of the Hill plots (Figure 28) indicated that there was little or no cooperativity for this substrate. When PEP was varied there seemed to be a departure from Michaelis-Menten kinetics (Figure 29). This became more apparent in the Hill plots (Figure 30), where n = 1.4. Tryptophan reduces this slope to n = 1.3.

DAHP synthetase (phe) appears to follow Michaelis-Menten kinetics when E-4-P is varied (Figure 31). Even in the presence of inhibitor there was no indication of sigmoidal kinetics. Hill plots (Figure 32) support this with slopes close to 1. This was not the case when PEP was varied (Figure 33). In the absence of phenylalanine, there was a positive cooperativity between the substrate binding sites with a Hill plot value of 1.8 (Figure 34). However, when phenylalanine was present this cooperativity seemed to be reduced, with n equal to 1.4.

Since non-Michaelis-Menten behavior was observed for some of the isozymes, substrate affinities were expressed as substrate concentrations for half-maximal velocity, (S) 0.5 v, rather than Km (4, 66). In each case the substrate affinity was altered only slightly by the presence of inhibitor (Table 27). The V max's were determined by extrapolation of the linear portion of the reciprocal plots at high

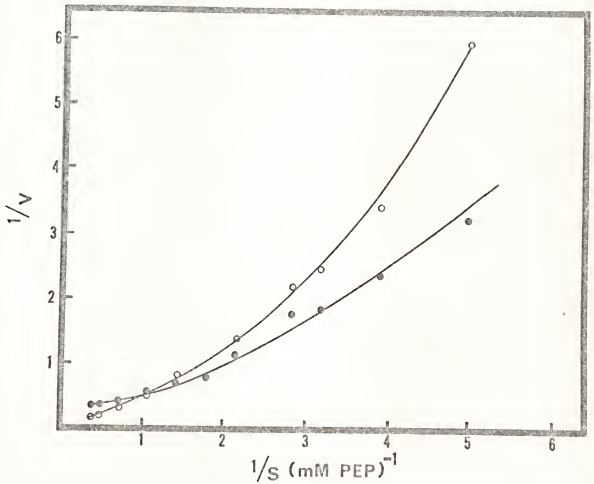

Figure 25. Double reciprocal plot of initial velocity (v) versus phosphoenolpyruvate concentration for DAHP synthetase (tyro) in the presence and absence of tyrosine. DAHP synthetase (tyro) which was eluted from DE-32 cellulose and rechromatographed on hydroxylapatite was used. The isozyme was present in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Erythrose-4-phosphate was present at a final concentration of 2 mM. The reaction mixtures were incubated 10 minutes at 37 C. The assay was as described in Materials and Methods. Tyrosine was prepared in the same buffer as the isozyme and present at a final concentration of 3×10^{-5} M which gave approximately 50% inhibition at saturating levels of substrates. Symbols: no tyrosine (○), 3×10^{-5} M tyrosine (●).

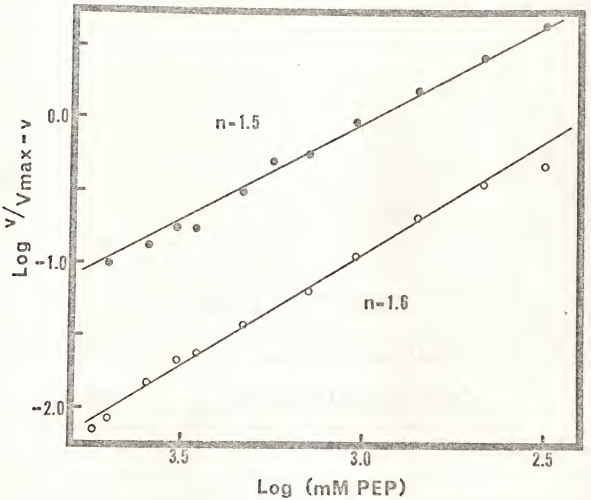

Figure 26. Hill plot of DAHP synthetase (tyro) activity at varied concentrations of phosphoenolpyruvate in the presence and absence of tyrosine. The data used in these calculations is the same as that recorded for Figure 25. Vmax was obtained from extrapolation of the linear portion (four points) at high concentrations of phosphoenolpyruvate in the reciprocal plot, v is the initial velocity. Symbols: no tyrosine (○), 3 x 10^{-5} M tyrosine (●).

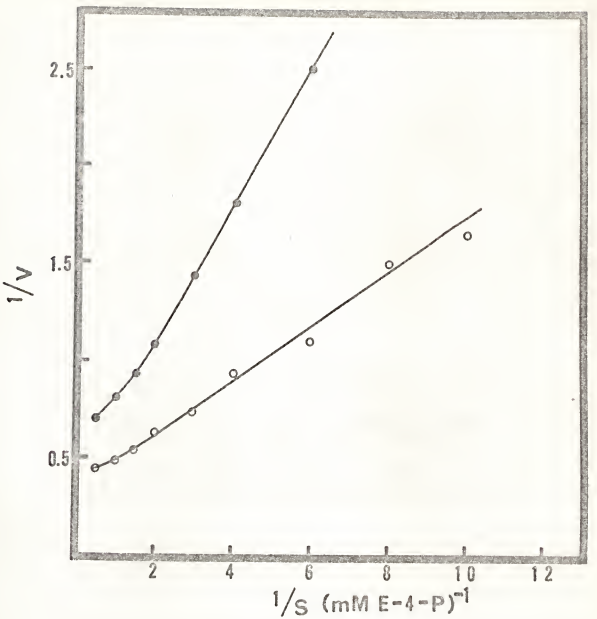

Figure 27. Double reciprocal plot of initial velocity (v) versus erythrose-4-phosphate concentrations for DAHP synthetase (tryp) in the presence and absence of tryptophan. DAHP synthetase (tryp) which was eluted from DE-32 cellulose and rechromatographed on hydroxylapatite was used. The isozyme was present in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Phosphoenolpyruvate was present at a final concentration of 2.8 mM. The reaction mixtures were incubated at 37 C for 10 minutes. The assay was as described in Materials and Methods. Tryptophan was prepared in the same buffer as the isozyme and present at a final concentration of 2×10^{-5} M, which gave approximately 50% inhibition at saturating levels of substrates. Symbols: no tryptophan (o), 2×10^{-5} M tryptophan (●).

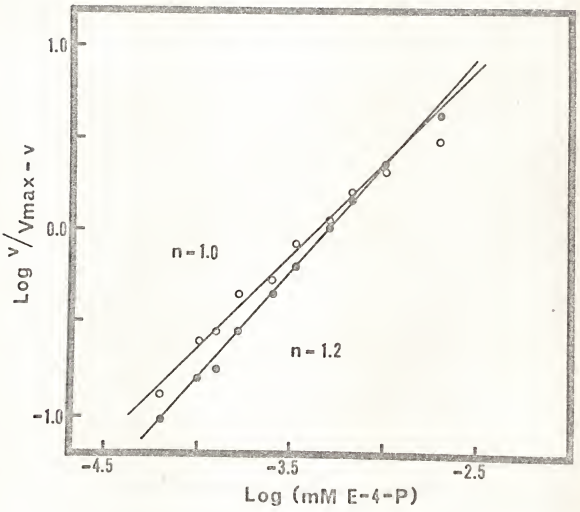

Figure 28. Hill plot of DAHP synthetase (tryp) activity at varied erythrose-4-phosphate concentrations in the presence and absence of tryptophan. The data used in these calculations is the same as that recorded for Figure 27. Vmax was obtained from extrapolation of the linear portion (four points) at high concentrations of erythrose-4-phosphate in the reciprocal plot, v is the initial velocity. Symbols: no tryptophan (○), 2×10^{-5} M tryptophan (●).

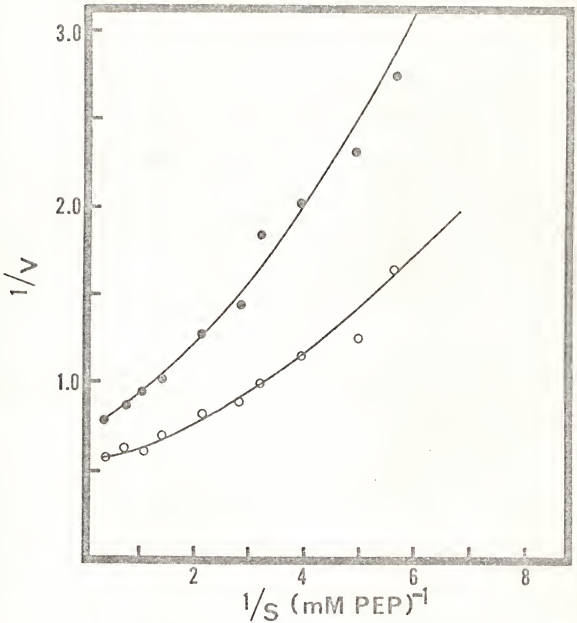

Figure 29. Double reciprocal plot of initial velocity (v) versus phosphoenolpyruvate concentration for DAHP synthetase (tryp) in the presence and absence of tryptophan. DAHP synthetase (tryp) which was eluted from DE-32 cellulose and rechromatographed on hydroxylapatite was used. The isozyme was present in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Erythrose-4-phosphate was present at a final concentration of 2 mM. The reaction mixtures were incubated 10 minutes at 37 C. The assay was as described in Materials and Methods. Tryptophan was prepared in the same buffer as the isozyme and present at a final concentration of 2×10^{-5} M which gave approximately 50% inhibition at saturating levels of substrates. Symbols: no tryptophan (O), 2×10^{-5} M tryptophan (●).

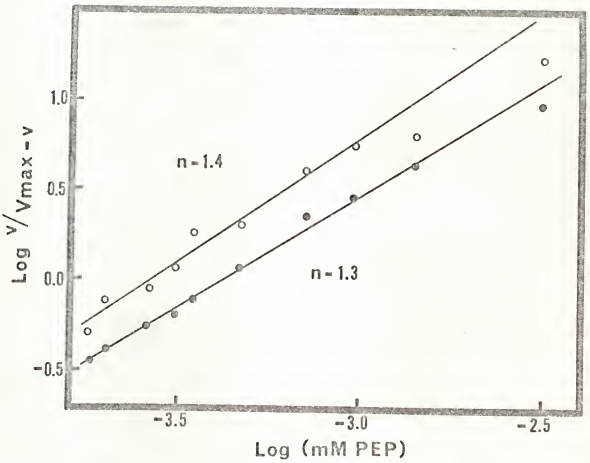

Figure 30. Hill plot of DAHP synthetase (tryp) activity at varied concentrations of phosphoenolpyruvate in the presence and absence of tryptophan. The data used in these calculations is the same as that recorded for Figure 29. Vmax was obtained from extrapolation of the linear portion (four points) at high concentrations of phosphoenolpyruvate in the reciprocal plot, v is the initial velocity. Symbols: no tryptophan (O), 2×10^{-5} M tryptophan (●).

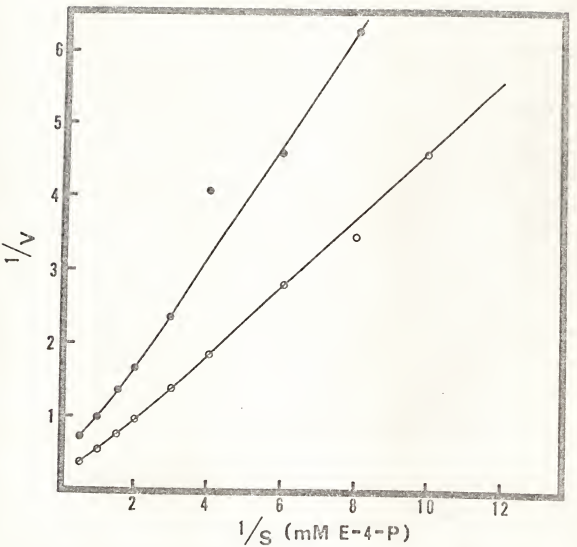

Figure 31. Double reciprocal of initial velocity (v) versus erythrose-4-phosphate concentrations for DAHP synthetase (phe) in the presence and absence of phenylalanine.
DAHP synthetase (phe) which was eluted from DE-32 cellulose and rechromatographed on hydroxylapatite was used. The isozyme was present in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Phosphoenolpyruvate was present at a final concentration of 2.8 mM. The reaction mixtures were incubated at 37 C for 10 minutes. The assay was as described in Materials and Methods. Phenylalanine was prepared in the same buffer as the isozyme and present at a final concentration of 3×10^{-6} M, which gave approximately 50% inhibition at saturating levels of substrates. Symbols: no phenylalanine (○), 3×10^{-6} M phenylalanine (●).

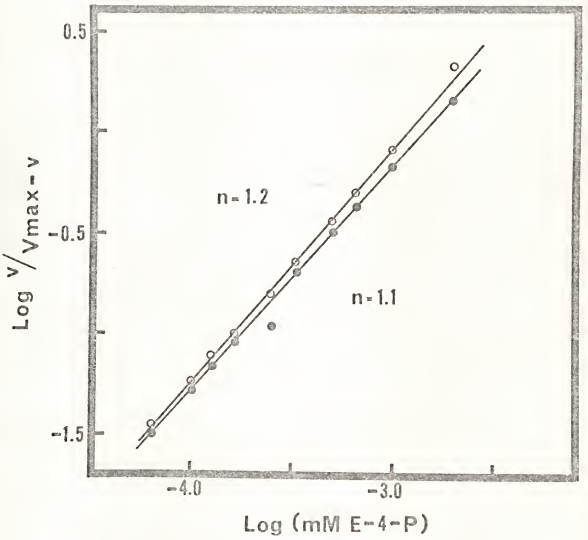

Figure 32. Hill plot of DAHP synthetase (phe) activity at varied erythrose-4-phosphate concentrations in the presence and absence of phenylalanine. The data used in these calculations is the same as that recorded for Figure 31. Vmax was obtained from extrapolation of the linear portion (four points) at high concentrations of erythrose-4-phosphate in the reciprocal plot, v is the initial velocity. Symbols: no phenylalanine (○), 3×10^{-6} M phenylalanine (●).

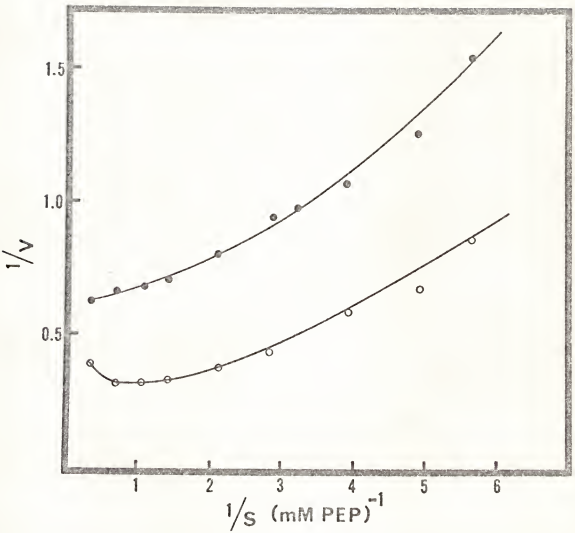

Figure 33. Double reciprocal plot of initial velocity (v) versus phosphoenolpyruvate concentration for DAHP synthetase (phe) in the presence and absence of phenylalanine. DAHP synthetase (phe) which was eluted from DE-32 cellulose and rechromatographed on hydroxylapatite was used. The isozyme was present in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Erythrose-4-phosphate was present at a final concentration of 2 mM. The reaction mixtures were incubated 10 minutes at 37 C. The assay was as described in Materials and Methods. Phenylalanine was prepared in the same buffer as the isozyme and present at a final concentration of 3×10^{-6} M which gave approximately 50% inhibition at saturating levels of substrates. Symbols: no phenylalanine (○), 3×10^{-6} M phenlalanine (●).

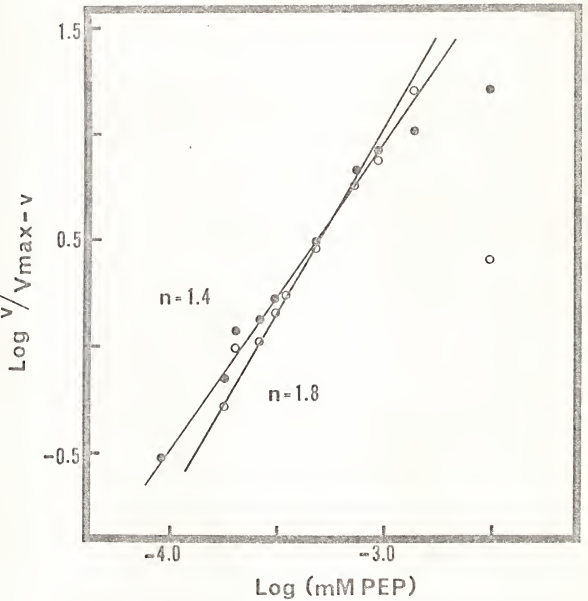

Figure 34. Hill plot of DAHP synthetase (phe) activity at varied concentrations of phosphoenolpyruvate in the presence and absence of phenylalanine. The data used in these calculations is the same as that recorded for Figure 33. Vmax was obtained from extrapolation of the linear portion (four points) at high concentrations of phosphoenol-pyruvate in the reciprocal plot, v is the initial velocity. Symbols: no phenylalanine (○), 3×10^{-6} M phenylalanine (●).

Table 27. Substrate affinities and maximum velocities of partially purified DAHP synthetase isozymes in the presence and absence of feedback inhibitors

DAHP Synthetase Isozyme	Effector	Substrate Affinity (S) 0.5 v		V max (mμM DAHP produced/min)	
		E-4-P (mM)	PEP (mM)	E-4-P	PEP
Tyro	--	1.2	7.9	8.0	15.0
Tyro	Tyrosine	0.8	5.8	3.5	4.2
Tryp	--	0.9	1.5	2.5	2.3
Tryp	Tryptophan	1.0	1.2	1.8	1.5
Phe	--	2.8	1.0	8.2	3.5
Phe	Phenylalanine	2.5	1.0	4.4	1.7

Inhibitors were present at the final concentrations noted in Figures 23, 27 and 31. V max was obtained by extrapolation of the linear portion (at least four points at high substrate concentrations) of the reciprocal plots. (S) 0.5 v values were then determined graphically at 1/2 V max.

concentrations of substrates. These values were significantly altered by the presence of inhibitor in all cases examined. Therefore the isozymes would appear to belong to the V system of allosteric enzymes (91).

Inhibition of the isozymes as a function of their inhibitor concentrations was demonstrated, as shown in Figures 35, 37, and 39. A plot of this data in terms of the modified Hill equation, $\log v - v_{SAT}/Vo - v = n' \log [I] - \log K$ according to Atkinson (4), is shown in Figures 36, 38 and 40; v and v_o being the initial reaction velocities in the presence and absence of inhibitor and v_{SAT} being the initial reaction velocity in the presence of a saturating concentration of inhibitor.

DAHP synthetase (tyro) was maximally inhibited by tyrosine at concentrations of 6×10^{-4} M (Figure 35) and the inhibition curve was sigmoidal indicating interaction between tyrosine binding sites. A Hill plot (Figure 36) of this data gave a slope of 1.8, while a plot of activity when all three amino acids were present in combination had a slope of 1.4. There was little or no inhibition by tryptophan or phenylalanine alone at any of the concentrations tested.

DAHP synthetase (tryp) was inhibited maximally (75%) by tryptophan at a concentration of 6×10^{-4} M (Figure 37). All three amino acids in combination gave a slightly higher inhibition than tryptophan alone at the lower concentrations tested. These data indicate some enzyme cooperativity for tryptophan binding, with an n' value of 1.4 (Figure 38). This was also true for the plot determined when the enzyme activity was assayed in the presence of all three of the aromatic amino acids.

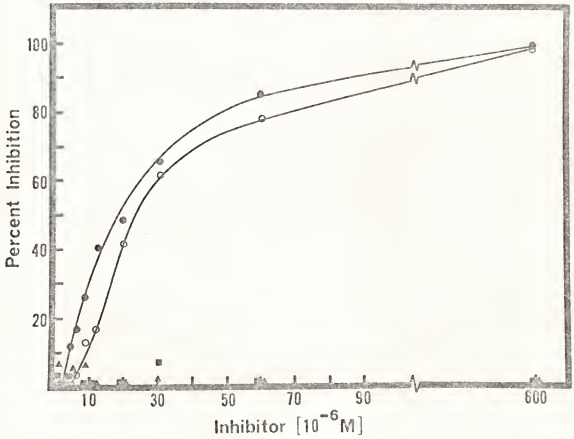

Figure 35. Inhibition of DAHP synthetase (tyro) by various concentrations of the aromatic amino acids. DAHP synthetase (tyro) eluted from DE-32 cellulose and rechromatographed on hydroxylapatite was used. Amino acids were prepared in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Isozyme was incubated with amino acid effector at the concentrations tested for 10 minutes at 4 C. Methods were as described in the text. Symbols: Tyrosine (○), Tryptophan (■), Phenylalanine (▲), Aro (●).

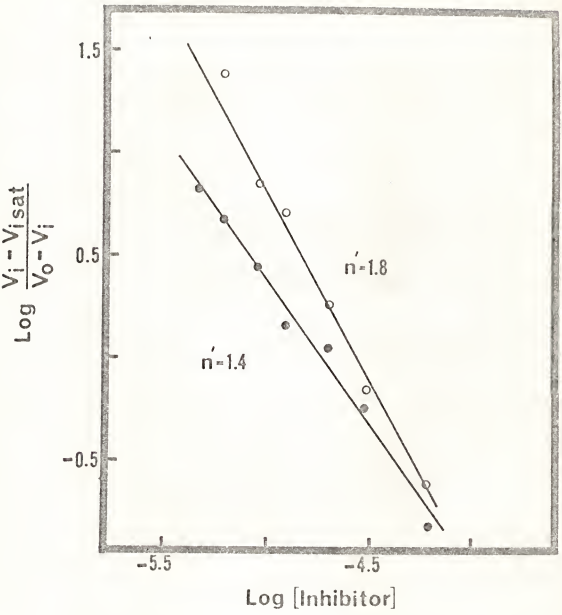

Figure 36. Hill plot of DAHP synthetase (tyro) inhibition by tyrosine and aro. The data used in these calculations is the same as that recorded for Figure 35. Initial reaction velocities are v and v_o, in the presence and absence of inhibitor; v_{SAT} is the initial reaction velocity in the presence of a saturating concentration of inhibitor; I is the inhibitor concentration; n' is the value of the Hill slope. Methods were as described in the text. Symbols: Tyrosine (○), Aro (●).

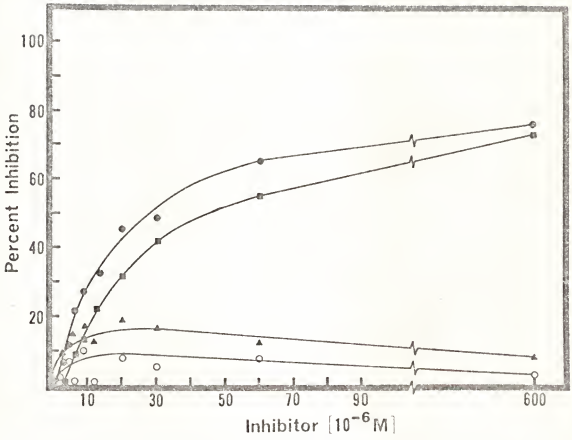

Figure 37. Inhibition of DAHP synthetase (tryp) by various concentrations of the aromatic amino acids. DAHP synthetase (tryp) eluted from DE-32 cellulose and rechromatographed on hydroxylapatite was used. Amino acids were prepared in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Isozyme was incubated with amino acid effector at the concentrations tested for 10 minutes at 4 C. Methods were as described in the text. Symbols: Tryptophan (■), Tyrosine (○), Phenylalanine (▲), Aro (●).

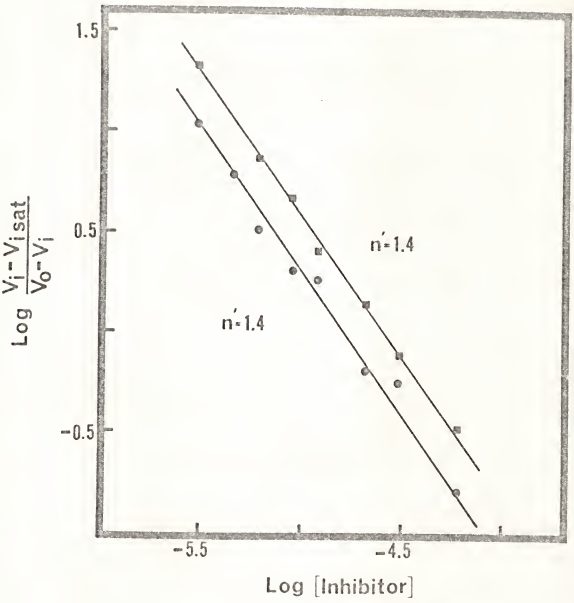

Figure 38. Hill plot of DAHP synthetase (tryp) inhibition by tryptophan and aro. The data used in these calculations is the same as that recorded for Figure 37. Initial reaction velocities are v and v_o, in the presence and absence of inhibitor; v_{SAT} is the initial reaction velocity in the presence of a saturating concentration of inhibitor; I is the inhibitor concentration; n' is the value of the Hill slope. Symbols: Tryptophan (■), Aro (●).

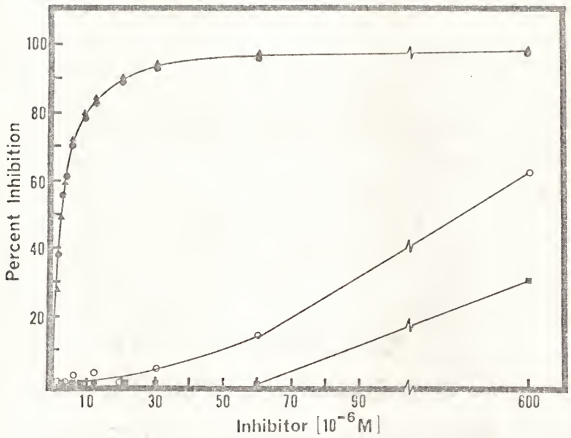

Figure 39. Inhibition of DAHP synthetase (phe) by various concentrations of the aromatic amino acids. DAHP synthetase (phe) eluted from DE-32 cellulose and rechromatographed on hydroxylapatite was used. Amino acids were prepared in 0.05 M potassium phosphate buffer, pH 7.0 + 0.1 mM $CoCl_2$. Isozyme was incubated with amino acid effector at the concentrations tested for 10 minutes at 4 C. Methods were as described in the text. Symbols: Tyrosine (○), Tryptophan (■), Phenylalanine (▲), Aro (●).

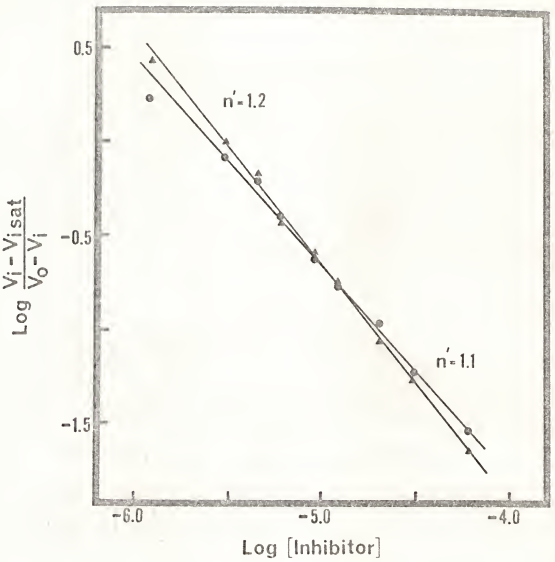

Figure 40. Hill plot of DAHP synthetase (phe) inhibition by phenylalanine and aro. The data used in these calculations is the same as that recorded for Figure 39. Initial reaction velocities are v and v_o, in the presence and absence of inhibitor; v_{SAT} is the initial reaction velocity in the presence of a saturating concentration of inhibitor; I is the inhibitor concentration; n' is the value of the Hill slope. Symbols: Phenylalanine (▲), Aro (●).

DAHP synthetase (phe) was sensitive to low concentrations of phenylalanine (Figure 39). The inhibition curve determined in the presence of all three aromatic amino acids coincided with that determined when only phenylalanine was present. Figure 39 appeared to be a rectangular hyperbola. However, a Hill plot (Figure 40) slope of 1.2 may indicate some cooperativity for this enzyme. Tyrosine became an effective inhibitor at concentrations above 6×10^{-5} M, as did tryptophan. At a concentration of 6×10^{-4} M tyrosine and tryptophan inhibited DAHP synthetase (phe) activity 63 and 32%, respectively.

DISCUSSION

DAHP synthetase, the first enzyme activity unique to the synthesis of the aromatic amino acids, was extracted from the marine bacterium Vibrio MB22 and partially characterized. Although the isozymes of DAHP synthetase studied here have not been purified to a homogeneous state, the complete separation between the three allowed examination of many of the properties of each isozyme without interference by the others.

Separation of the three isozymes was achieved by using DEAE cellulose chromatography. Each of the three isozymes was primarily inhibited by one of the aromatic amino acids, tyrosine, phenylalanine or tryptophan. The tyrosine sensitive isozyme activity was dominant in that it made up 60% of the total activity, while DAHP synthetase (tryp) and DAHP synthetase (phe) corresponded to 30% and 10% of the total activity, respectively. This type of inhibition may be non-specific since tyrosine and tryptophan, in contrast to phenylalanine, are effective inhibitors only at the highest concentrations tested. It is also significant that leucine at the highest concentration inhibited this isozyme activity to the same extent as did tryptophan. Tyrosine is structurally very similar to phenylalanine, and may influence enzyme activity by binding to an effector site which is not entirely specific for phenylalanine. On the other hand, DAHP synthetase (phe) was found to have an apparent molecular weight nearly three times larger than either of the other two isozymes. This may indicate an aggregate of

isozyme molecules each having an active site and a different effector site. The cooperativity observed for PEP binding supports multiple active sites, and the Hill plot for phenylalanine inhibition indicated no cooperativity or a single binding site for this effector. Since both DAHP synthetase (tyro) and DAHP synthetase (tryp) showed cooperativity for PEP binding and have significantly smaller molecular weights, it is conceivable that the phenylalanine sensitive isozyme is similar to these isozymes and is aggregated with subunits that only have effector sites for tyrosine and tryptophan.

DAHP synthetase isozymes separated by DEAE cellulose were found to be activated by divalent cations, especially cobalt and manganese, after treatment with EDTA. These divalent cations were found to affect not only catalytic activity but the sensitivity of the isozymes to their end product inhibitors. Activation of this enzyme by cobalt has been previously reported for some non-marine organisms (33, 89, 93, 104, 121, 133, 137). The physiological significance of divalent cations in regulation has not been systematically examined as yet, although their effect has been noted (128). They are probably involved in the binding of substrate to the enzyme and may play an important role in determining the secondary, tertiery and quaternary structures of regulatory enzymes. The effects of divalent cations on the isozymes of DAHP synthetase from Vibrio MB22 may be a reflection of interaction at or close to the catalytic site since PEP, one of the substrates, protects all the isozymes from inactivation by EDTA. However, since the presence of some divalent cations also affects isozyme sensitivity to inhibitors, a configurational change in the protein which causes a change in the effector and catalytic site may be indicated. It must be noted that

the ions chelated by EDTA may not necessarily be the same ions that are added back for activation. Whether these divalent cations are involved in substrate binding or enzyme configuration has not been determined. It would be interesting to investigate the effects of divalent cations on the kinetic properties of these isozymes.

Chromatography of DEAE cellulose separated isozymes on hydroxylapatite caused a marked change in both their properties of being inactivated by EDTA and activated by divalent cations, as well as their stability. Hydroxylapatite, which is composed of calcium phosphate, chelates divalent cations. This chelation may or may not be directly involved in the alteration of the isozymes in some way. Although incubation of the isozymes with the protein fractions removed by this procedure did not reestablish the property of divalent cation activation, it is still possible that removal of some proteins normally associated with the isozymes may be the cause of this phenomenon. Some conformational change in the enzyme could cause the cation binding site to be less accessible to EDTA or added cations.

As mentioned above, chromatography of the isozymes on hydroxylapatite caused them to be less stable under the conditions tested. The loss of stability may also be due to the removal of proteins which are normally loosely associated with the enzyme and form a weak complex in vivo. Some of these proteins may act non-specifically to partially protect the enzyme, as did bovine serum albumin. However, some proteins may be specific as indicated by the results obtained with one of the protein fractions which gave greater stability than higher concentrations of bovine serum albumin. Complete stabilization under the conditions used was achieved with the addition of phosphoenolpyruvate,

one of the substrates. However, since the supernatant of the boiled protein fraction II, which was the most effective stabilizer, also stabilizes somewhat, it may be that a non-protein molecule is involved in this stabilization and acts in a manner similar to phosphoenolpyruvate.

It is interesting to note that monovalent cations were slightly inhibitory to the isozymes in vitro, since the sodium chloride concentration used in the growth medium was four times greater than that tested in vitro. If the levels of Na^+, K^+ and Cl^- are the same extracellularly and intracellularly in Vibrio MB22 as reported for a marine Pseudomonad (136), then the isozymes are either protected from the effects of monovalent cations by compartmentalization or the effects of these cations are different in vivo.

Monod, Changeux and Jacob (90) have emphasized the fact that metabolite effectors of regulatory enzymes are not isosteric but rather allosteric (i.e., they have no structural resemblance) with respect to the normal substrates of the enzyme. The allosteric effector binding sites are also separable from substrate binding sites (44). The three DAHP synthetase isozymes of Vibrio MB22 are therefore allosteric, since they are feedback regulated by their aromatic amino acid end products.

Investigation of the initial reaction velocities of these three isozymes at varied substrate concentrations yielded double reciprocal plots which showed an upward curvature at higher substrate concentrations. This is a departure from classical Michaelis-Menten kinetics. This cooperativity was observed for all three isozymes when phosphoenolpyruvate was varied and only for DAHP synthetase (tyro) with variable erythrose-4-phosphate. Cooperativity became more apparent when the data was examined in the form of Hill plots which indicate interaction

of binding sites when the slope of the plot is greater than 1 (4). This was especially evident in the Hill plots of isozyme inhibition by the amino acid end products. These positive homotropic effects indicate the existence of two or more interacting substrate or effector binding sites on the enzyme molecule, such that the binding of one molecule facilitates the binding of the next.

Until recently, substrate kinetics of DAHP synthetase from most non-marine organisms showed no cooperativity for either substrate (41, 48, 62, 93, 131, 132, 155). However, Doy (33) using crude extracts of Neurospora crassa demonstrated homotropic cooperativity for both substrates. His Hill plot values however, were reduced to near unity in the presence of the end product inhibitors. This effect on cooperativity by the inhibitors was also observed for the isozymes of Vibrio MG22. Isozymes of Sacchromyces cerevisiae (137) separated by affinity chromatography exhibited non-Michaelis-Menten kinetics for both substrates, contrary to a previous report by Lingens et al. (72), who separated the same isozymes by ammonium sulfate fractionation. Takahashi and Chan (137) suggested, in this case, that ammonium sulfate treatment may affect the isozymes causing them to lose substrate cooperativity, while a milder method of separation, affinity chromatography, would preserve this property. It is therefore important to consider the methods used for preparing an enzyme when examining its characteristics. In all DAHP synthetase enzymes where it was examined, inhibition kinetics were found to be cooperative (30, 33, 41, 62, 93, 137).

Cooperativity of substrate and effector binding is a common feature for a large number of regulatory enzymes (90). There are a number of ways by which sigmoidal kinetics of regulatory enzymes can be explained.

However, two models are most popular. The symmetry model of Monod et al. (91) takes into account the fact that enzymes are generally composed of two or more identical subunits arranged in a symmetrical manner, and this symmetry is conserved as the enzyme fluctuates between two or more conformers with differing affinity for substrate molecules and allosteric effector molecules. Koshland et al. (66) attempted to explain kinetic and regulatory properties of proteins by an 'induced-fit' theory. This model proposes that binding of a ligand at one site increases or decreases the affinity of a ligand at another site, which in turn affects another and so on. Cooperativity for both substrates and effector ligands in the DAHP synthetase isozymes of Vibrio MB22 is contrary to predictions of the equilibrium model of Monod et al. (91) for V system enzymes, but such kinetic behavior can be accommodated in the induced conformational change model.

The sigmoidal responses of enzyme activity to increasing substrate or effector concentrations is probably of fundamental importance in cellular regulation. There exists a threshold concentration below which the enzyme activity is relatively insensitive to variations in concentrations of substrates and effectors, and above which relatively slight changes in concentration evoke marked effects in enzymic activity (15). The sigmoidal saturation function restricts the metabolic control to a very narrow, selected range of substrate and effector concentrations and should thereby facilitate a more rigorous control of enzymic activity. A selective advantage for the organism may be gained by the selection of regulatory enzymes having cooperative saturation functions.

Because of the non-Michaelis-Menten behavior of substrate kinetics in the presence and absence of effectors, substrate affinities are

expressed as substrate concentrations for half-maximal velocity, $(S)_{0.5v}$ rather than Km (4, 66). In all the cases investigated for these three isozymes of Vibrio MB22, the V max's were significantly altered while the substrate affinities did not vary appreciably. This would suggest non-competitive inhibition by the effector, or binding of the end product to an inhibitor specific site on the enzyme resulting in the induction or stabilization of a conformational state that has a lower catalytic potential. However, the terms "competitive" and "non-competitive" inhibition to describe the effects of metabolites on allosteric enzymes are avoided by using the nomenclature introduced by Monod et al. (91). The non-competitive system is referred to as the "V system" describes the opposite situation. The three isozymes of DAHP synthetase in this organism therefore belong to the "V system" of allosteric inhibition. DAHP synthetases from some non-marine organisms have also been shown to be "V system" enzymes (62, 91, 155).

In conclusion, the feedback control of DAHP synthetase isozymes from Vibrio MB22 appears to be similar to that found for some non-marine organisms (30, 31, 47, 58, 59, 152, 153). Some properties of these isozymes as divalent cation activation, stabilization, pH effects, molecular weights, substrate and inhibition kinetics of the three DAHP synthetase isozymes have been examined. However, there are many problems which remain to be investigated, such as the specific effect of divalent cations on catalytic activity and inhibitions, the affect of hydroxylapatite chromatography on divalent cation activation and stability, the possibility of isozyme aggregation, as well as the role of divalent cations in homo- and hetero-molecular kinetic interactions.

LITERATURE CITED

1. Ames, B. N., R. G. Martin and B. J. Garry. 1961. The first step in histidine biosynthesis. J. Biol. Chem. 236:2019-2026.

2. Andrews, P. 1964. Estimation of the molecular weights of proteins by sephadex gel filtration. Biochem. J. 91:222-232.

3. _____. 1965. The gel-filtration behavior of proteins related to their molecular weights over a wide range. Biochem. J. 96:595-606.

4. Atkinson, D. E. 1966. Regulation of enzyme activity. Ann. Rev. Biochem. 35:85-124.

5. _____. 1969. Regulation of enzyme function. Ann. Rev. Microbiol. 23:47-68.

6. Brooke, M. S., D. Ushiba and B. Magasanik. 1954. Some factors affecting the excretion of orotic acid by mutants of Aerobacter aerogenes. J. Bacteriol. 68:534-540.

7. Brown, K. D. and C. H. Doy. 1963. End-product regulation of the general aromatic pathway in Escherichia coli W. Biochim. Biophys. Acta. 77:170-172.

8. _____ and _____. 1966. Control of 3 isozymic 7-phospho-2-oxo-3-deoxy-D-arabino-heptulosonate-D-erythrose-4-phosphate lyases of Escherichia coli W and derived mutants by repressive and "inductive" effects of the aromatic amino acids. Biochim. Biophys. Acta. 118:157-172.

9. _____ and W. K. Maas. 1966. Control of aromatic biosynthesis in Escherichia coli K-12. Fed. Proc. 25:338.

10. Burton, K. 1959. Formation constants for the complexes of adenosine di- or tri- phosphate with magnesium or calcium ions. Biochem. J. 71:388-395.

11. Caffey, J. J. 1967. Induceable synthesis of bacterial luciferase: specificity and kinetics of induction. J. Bacteriol. 94:1638-1647.

12. Caskey, T. C., D. M. Ashton and J. B. Wyngarden. 1964. The enzymology of feedback inhibition of glutamine phosphoribosyl-pyrophosphate amidotransferase by purine ribonucleotides. J. Biol. Chem. 239:2570-2579.

13. Chance, B. and B. C. Maehly. 1955. Assay of catalases and peroxidases. In: Methods in Enzymology. S. P. Colowick and N. O. Kaplan, eds. Academic Press, New York 2:764.

14. Chandra, P. and L. C. Vining. 1968. Conversion of phenylalanine and tyrosine by microorganisms. Can. J. Microbiol. 14:573-578.

15. Changeux, J. P. 1961. Allosteric interactions on biosynthetic L-threonine deaminase from Escherichia coli K-12. Cold Spring Harbor Symp. Quant. Biol. 28:497-504.

16. Cohen, G. N. 1965. Regulation of enzyme activity in microorganisms. Ann. Rev. Microbiol. 19:105-126.

17. _____. 1969. The aspartokinases and homoserine dehydrogenases of Escherichia coli. In: Current topics in cellular regulation, B. L. Horecker and E. R. Stadtman (ed.). Vol. 1. Academic Press Inc., New York.

18. Costerton, J. W., C. F. Forsberg, T. I. Matula, F. L. A. Buckmire, and R. A. MacLeod. 1967. Nutrition and metabolism of marine bacteria. XVI. Formation of protoplasts, spheroplasts and related forms from a gram-negative marine bacterium. J. Bacteriol. 94:1764-1777.

19. Cotton, R. G. H. and F. Gibson. 1965. The biosynthesis of phenylalanine and tyrosine; enzyme converting chorismic acid into prephenic acid and their relationships to prephenate dehydratase and prepherate dehydrogenase. Biochim. Biophys. Acta. 100:76-88.

20. Crisley, F. D. 1963. Effect of sodium chloride on growth, glucose utilization and acid production in Proteus vulgaris. J. Bacteriol. 86:346-347.

21. Datta, P. 1966. Purification and feedback control of threonine deaminase activity in Rhodopseudomonas spheroides. J. Biol. Chem. 241:5836-5844.

22. _____. 1966. Regulation of branched biosynthetic pathways in bacteria. Science 165:556-562.

23. _____ and H. Gest. 1964. Alternative patterns of end product control in biosynthesis of amino acids of the aspartic family. Nature 203:1259-1261.

24. _____ and L. Prakash. 1966. Aspartokinase of Rhodopseudomonas spheroides. Regulation of enzyme activity of aspartate β-semialdehyde. J. Biol. Chem. 241:5827-5835.

25. Davis, B. D. 1950. Studies on nutritionally deficient bacterial mutants isolated by means of penicillin. Experentia 6:41-50.

26. DeHauwer, G. 1964. Studies of pyrroline dehydrogenase and of catabolism regulation of arginine and proline in Bacillus subtilis. Biochim. Biophys. Acta. 81:257-269.

27. Devoe, I. W. and E. L. Oginsky. 1969. Antagonistic effect of monovalent cations in maintenance of cellular integrity of a marine bacterium. J. Bacteriol. 98:1355-1367.

28. _____, J. Thompson, J. W. Costerton and R. A. MacLeod. 1970. Stability of comparative transport capacity of cells, mureinoplasts and true protoplasts of a gram-negative bacterium. J. Bacteriol. 101:1014-1026.

29. Doy, C. H. 1967. Tryptophan as an inhibitor of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase. Biochem. Biophys. Res. Commun. 26:187-192.

30. _____. 1967. 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthetase as an "allosteric" function and ligand interactions with this enzyme from Neurospora crassa. Biochem. Biophys. Res. Commun. 28:851-856.

31. _____. 1968. Aromatic biosynthesis in yeast: II. Feedback inhibition and repression of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase. Biochim. Biophys. Acta. 151:293-295.

32. _____. 1968. Control of aromatic biosynthesis particularly with regard to the common pathway and the allosteric enzyme 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase. Rev. Pure and Appl. Chem. 18:41-78.

33. _____. 1968. The nature of DAHP synthetase in extracts of wild type Neurospora crassa, a reaction controlled by two activating substrates and three allosteric negative modifiers. Biochim. Biophys. Acta. 159:352-365.

34. _____. 1970. Isozymes and allosteric inhibition of Neurospora crassa 3-deoxy-D-arabino-heptulosonate 7-phosphate synthase. Biochim. Biophys. Acta. 198:364-375.

35. _____ and K. D. Brown. 1965. Control of aromatic biosynthesis: the multiplicity of 7-phospho-2-oxo-3-deoxy-D-arabino-heptonate D-erythrose-4-phosphate-lyase pyruvate phosphorylating in Escherichia coli W. Biochim. Biophys. Acta. 104:377-389.

36. _____ and J. M. Cooper. 1966. Aromatic biosynthesis in yeast: I. The synthesis of tryptophan and the regulation of this pathway. Biochim. Biophys. Acta. 127:302-316.

37. Drapeau, G. R. and R. A. MacLeod. 1963. Nutrition and metabolism of marine bacteria. XII. Ion activation of adenosine triphosphatase in membranes of marine bacterial cells. J. Bacteriol. 85:1413-1419.

38. _____ and R. A. MacLeod. 1963. Na^+ dependent active transport of α-aminoisobutyric acid into cells of a marine pseudomonad. Biochem. Biophys. Res. Commun. 12:111-115.

39. _____, T. I. Matula and R. A. MacLeod. 1966. Nutrition and metabolism of marine bacteria. XV. Relation of Na^+ activated transport to the Na^+ requirement of a marine pseudomonad for growth. J. Bacteriol. 92:63-71.

40. Eagon, R. G. and C. H. Wong. 1962. Dissimilation of glucose and gluconic acid by Pseudomonas natriegens. J. Bacteriol. 83:879-886.

41. Eberspacher, J., H. Uesseler and F. Lingens. 1970. Eigenschaften der phospho-2-oxo-3-desoxyheptonat-aldolase aus Claviceps SD58. Hoppe-Seyler's Z. Physiol. Chem. 351:1465-1474.

42. Forsberg, C. W., J. W. Costerton and R. A. MacLeod. 1970. Separation and localization of cell wall layers of a gram-negative bacterium. J. Bacteriol. 104:1338-1353.

43. Gamborg, O. L. 1966. Aromatic metabolism in plants. II. Enzymes of the skikimate pathway in suspension cultures of plant cells. Can. J. Biochem. 44:791-799.

44. Gerhardt, J. C. and A. B. Pardee. 1962. The enzymology of control of feedback inhibition. J. Biol. Chem. 237:891-896.

45. Gibson, M. I. and F. Gibson. 1964. Preliminary studies on the isolation and metabolism of an intermediate in aromatic biosynthesis: chorismic acid. Biochem. J. 90:248-256.

46. _____ and J. Pittard. 1968. Pathway of biosynthesis of aromatic amino acids and vitamins and their control in microorganisms. Bacteriol. Rev. 32:465-492.

47. Gollub, E., H. Zalkin and D. B. Sprinson. 1967. Correlation of genes and enzymes, and studies on regulation of the aromatic pathway in Salmonella. J. Biol. Chem. 242:5323-5328.

48. Görisch, H. and F. Lingens. 1971. 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase of Streptomyces aureofaciens Tu24. I. Partial purification and properties. Biochim. Biophys. Acta. 242:617-629.

49. Gots, J. S. 1950. Accumulation of 5 (4)-amino-4-(5)-imidazole-carboxamide in relation to sulfonamide bacteriostasis and purine metabolism in Escherichia coli. Fed. Proc. 9:178-179.

50. Gross, R. and N. W. Coles. 1968. Adenosine triphosphatase in isolated membranes of Staphylococcus aureus. J. Bacteriol. 95: 1322-1326.

51. Halsall, D. M., D. E. A. Catheside and C. H. Doy. 1971. Some properties of the 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase isozymes from mutant strains of Neurospora crassa. Biochim. Biophys. Acta. 227:464-472.

52. _____ and C. H. Doy. 1970. Studies concerning the biochemical genetics and physiology of activity and allosteric inhibition mutants of Neurospora crassa 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase. Biochim. Biophys. Acta. 185:432-446.

53. Happold, F. C. and A. Struyvenberg. 1954. The activation of tryptophanase apo-enzyme by potassium, ammonium and rubidium ions. Biochem. J. 58:379-382.

54. Harvey, E. N. 1915. Studies of light production by luminous bacteria. Amer. J. Physiol. 37:230-239.

55. Harvey, H. W. 1966. The chemistry and fertility of sea waters. Cambridge University Press, London, England. 187 pp.

56. Hayaski, M. and R. Uchida. 1965. A cation activated adenosine-triphosphatase in cell membranes of halophilic Vibrio parahaemolyticus. Biochim. Biophys. Acta. 110:207-209.

57. Hubbard, J. S. and E. R. Stadtman. 1967. Regulation of glutamine synthetase. VI. Interaction of inhibitors for Bacillus licheniformis glutamine synthetase. J. Bacteriol. 94:1016-1024.

58. Jensen, R. A. and D. S. Nasser. 1968. Comparative regulation of isozymic 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase in microorganisms. J. Bacteriol. 95:188-196.

59. _____, _____ and E. W. Nester. 1967. Comparative control of a branch-point enzyme in microorganisms. J. Bacteriol. 94:1582-1593.

60. _____ and E. W. Nester. 1965. The regulatory significance of intermediary metabolites: control of aromatic acid biosynthesis by feedback inhibition in Bacillus subtilis. J. Mol. Biol. 12:468-481.

61. _____ and _____. 1966. Regulatory enzymes of aromatic amino acid biosynthesis in Bacillus subtilis. I. Purification and properties of 3-deoxy-D-arabino-heptulosonate 7 phosphate synthetase. J. Biol. Chem. 241:3365-3372.

62. _____ and _____. 1966. Regulatory enzymes of aromatic amino acid biosynthesis in Bacillus subtilus. II. The enzymology of feedback inhibition of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase. J. Biol. Chem. 241:3373-3380.

63. Johnson, F. H. and E. N. Harvey. 1937. Osmotic and surface properties of marine luminous bacteria. J. Cell. Comp. Physiol. 9:363-380.

64. Kingdon, H. S., B. M. Shapiro and E. R. Stadtman. 1967. Regulation of glutamine synthetase. VIII. ATP: glutamine synthetase adenyltransferase an enzyme that catalyzes alterations in the regulatory properties of glutamine synthetase. Proc. Natl. Acad. Sci. U. S. 58:1703-1710.

65. Kornberg, A. 1955. Lactate dehydrogenase in muscle. In: Methods in Enzymology. S. P. Colowick and N. O. Kaplan, eds. Academic Press, New York. 1:441.

66. Koshland, D. E., G. Nemethy and D. Filmer. 1964. Comparison of experimental binding data and theoretical models in proteins containing subunits. Biochem. 5:365-385.

67. Kratz, W. A. and J. Myers. 1955. Nutrition and growth of several blue-green algae. Amer. J. Botany 42:282-287.

68. Lacroute, F., A. Pierard, M. Grenson and J. M. Wiame. 1965. The biosynthesis of carbamyl phosphate in Saccharomyces cerevisiae. J. Gen. Microbiol. 40:127-142.

69. Lee, N. M., E. M. Lansford and W. Shive. 1966. A study of the mechanism of lysine-sensitive aspartokinase control by lysine. Biochem. Biophys. Res. Commun. 24:315-318.

70. Lingens, F. 1968. The biosynthesis of aromatic amino acids and its regulation. Angew. Chem. Internat. Edit. 7:350-360.

71. _____, W. Goebel and H. Uesseler. 1966. Regulation der biosynthese der aromatischen aminosauren in Saccharomyces cerevisiae. I. Hemmung der enzymaktivitaten (feedback-werkung). Biochem. Z. 346:357-367.

72. _____, _____ and _____. 1967. Regulation der biosynthese der aromatischen aminosauren in Saccharomyces cerevisiae. Repression, induction and aktiverien. European J. Biochem. 1: 363-374.

73. _____, _____ and _____. 1967. Regulation der biosynthese der aromatischen aminosauren in Claviseps paspali. European J. Biochem. 2:442-447.

74. Lorence, J. and E. W. Nester. 1967. Multiple molecular forms of chorimate mutase in Bacillus subtilis. Biochem. 6:1541-1553.

75. Lowry, O. H., N. J. Rosebrough, A. L. Farr and R. J. Randall. 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem. 193:265-275.

76. MacLeod, R. A. 1965. The question of the existance of specific marine bacteria. Bacteriol Rev. 29:9-23.

77. _____, C. A. Claridge, A. Hori and J. F. Murray. 1958. Observations on the function of sodium in the metabolism of a marine bacterium. J. Biol. Chem. 232:829-834.

78. _____, A. Hori. 1960. Nutrition and metabolism of marine bacteria. VIII. Tricarboxylic acid cycle enzymes in a marine bacterium and their response to inorganic salts. J. Bacteriol. 80:464-471.

79. _____, _____ and S. M. Fox. 1963. Nutrition and metabolism of marine bacteria. X. The glyoxylate cycle in a marine bacterium. Can. J. Microbiol. 9:639-644.

80. _____ and T. I. Matula. 1961. Solute requirements for preventing lysis of some marine bacteria. Nature. 142:1209-1210.

81. _____ and _____. 1962. Nutrition and metabolism of marine bacteria. XI. Some characteristics of the lytic phenomenon. Can. J. Microbiol. 8:883-896.

82. _____ and E. Onofrey. 1955. Nutrition and metabolism of marine bacteria. II. Observations on the relation of the sea water to the growth of marine bacteria. J. Bacteriol. 71:661-667.

83. _____ and _____. 1957. Nutrition and metabolism of marine bacteria. III. The relation of sodium and potassium to growth. J. Cell. Comp. Physiol. 50:389-401.

84. _____ and _____. 1957. Nutrition and metabolism of marine bacteria. VI. Quantitative requirements for halids, magnesium, calcium and iron. Can. J. Microbiol. 3:753-759.

85. Main, R. K., M. J. Wikens and L. J. Cola. 1959. A modified calcium phosphate for column chromatography of polynucleotides and proteins. J. Amer. Chem. Soc. 81:6490-6495.

86. Martin, R. G., B. N. Ames and B. J. Garry. 1961. The mechanism of feedback inhibition in histidine biosynthesis. Fed. Proc. 20:225.

87. Matula, T. I., V. S. Srivastana, P. Wong and R. A. MacLeod. 1970. Transport and retention of K^+ and other metabolites in a marine pseudomonad and their relation to the mechanism of optical effects. J. Bacteriol. 102:790-796.

88. Meuris, P., F. Lacroute and P. P. Slonimski. 1967. Etude systematique de mutants inhibes par leurs propes metabolites chez la levure Saccharomyces cerevisiae. I. Obtention et caractérisation des differentes classes de mutants. Genetics 56:149-161.

89. Moldovanyi, I. Sz. and G. Denes. 1968. Mechanism of the action and of the allosteric inhibition of 3-deoxy-D-arabino heptulosonate 7-phosphate synthetase (tyrosine-sensitive) of Escherichia coli W. Biochim. Biophys. Acad. Sci. Hung. 3:259-268.

90. Monod, J., J. P. Changeux and F. Jacob. 1963. Allosteric proteins and cellular control systems. J. Mol. Biol. 6:306-329.

91. _____, J. Wyman and J. P. Changeux. 1965. On the nature of allosteric transition: a plausible model. J. Mol. Biol. 12:88-118.

92. Moulton, E. W. 1971. Response of enzymes in the aromatic amino acid pathway of Vibrio MB-22 to varying end product concentrations. Thesis, University of Florida, Gainesville, Florida.

93. Nagano, H. and H. Zalkin. 1970. Tyrosine-inhibited 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase from Salmonella typhimurium. Arch. Biochem. Biophys. 138:58-65.

94. Nester, E. W. and R. A. Jensen. 1966. Control of aromatic acid biosynthesis in Bacillus subtilis sequential feedback inhibition. J. Bacteriol. 91:1594-1598.

95. _____, J. Lorence and D. S. Nasser. 1967. An enzyme aggregate involved in the biosynthesis of aromatic amino acids in Bacillus subtilis: Its possible function in feedback regulation. Biochem. 6:1553-1562.

96. Nierlich, D. P. and B. Magasanik. 1965. Regulation of purine ribonucleotide synthesis by end product inhibition. The effect of adenine and guanine ribonucleotides on the 5'-phosphoribosyl-pyrophosphate amidotransferase of Aerobacter aerogenes. J. Biol. Chem. 240:358-365.

97. Nossal, P. M. 1951. The decarboxylation of malic acid by Lactobacillus arabinosus. Biochem. J. 49:409-413.

98. O'Brian, R. W. and J. R. Stern. 1969. Requirement for sodium in the anaerobic growth of Aerobacter aerogenes on citrate. J. Bacteriol. 98:388-393.

99. Patte, J. C., G. Lebras and G. N. Cohen. 1967. Regulation by methionine of the synthesis of a third aspartokinase and of a second homoserine dehydrogenase in Escherichia coli K-12. Biochim. Biophys. Acta. 136:245-257.

100. Paulus, H. and E. Gray. 1964. Multivalent feedback inhibition of aspartokinase in Bacillus polymyxa. J. Biol. Chem. 239:PC4008-4009.

101. Payne, W. J. 1958. Studies on bacterial utilization of uronic acids. III. Induction of oxidative enzymes in a marine isolate. J. Bacteriol. 76:301-307.

102. _____. 1960. Effects of sodium and potassium ions on growth and substrate penetration of a marine pseudomonad. J. Bacteriol. 80:696-700.

103. Piérard, A. 1966. Control of the activity of Escherichia coli carbamyl phosphate synthetase by antagonistic allosteric effectors. Science. 154:1572-1573.

104. Pittard, J., J. Camakaris and B. J. Wallace. 1969. Inhibition of 3-deoxy-D-arabinoheptulosonic acid 7- phosphate synthetase (trp) in Escherichia coli. J. Bacteriol. 97:1242-1247.

105. _____ and F. Gibson. 1970. The regulation of biosynthesis of aromatic amino acids and vitamins. In: Current topics in cellular regulation, B. L. Horecker and E. R. Stodtman (eds), Vol. 2. Academic Press Inc., New York.

106. Polly, S. M. 1964. The alkaline phosphatase activity of a marine bacterium. The effect of phosphates. Thesis, University of Florida, Gainesville, Florida.

107. Pratt, D. B. 1963. Specificity of the solute requirements by marine bacteria on primary isolation from sea water. Nature 199:1308.

108. _____ and M. Austin. 1961. Osmotic regulation of the growth rate of marine bacteria. Bacteriol. Proc. p. 47.

109. _____ and F. C. Happold. 1960. Requirements for indole production by cells and extracts of a marine bacterium. J. Bacteriol. 80:232-236.

110. Ramos, F., V. Stalon, A. Piérard and J. M. Wiame. 1967. The specialization of the two ornithine carbamyltransferases of Pseudomonas. Biochim. Biophys. Acta. 139:98-106.

111. Richards, J. H. and J. B. Hendrickson. 1964. The biosynthesis of steroids, terpenes and acetogenins. W. A. Benjamin, Inc., New York.

112. Rhodes, M. E. and W. J. Payne. 1962. Further observations on effects of cations on enzyme induction in a marine bacterium. Antonie van Leuvenhoek J. Microbiol. Sero. 28:302-314.

113. Roberts, R. B., P. H. Abelson, D. B. Cowie, E. T. Bolton and R. J. Britten. 1955. Studies of biosynthesis in Escherichia coli. Carnegie Inst. Wash. Publ. No: 607, Washington, D. C.

114. Salter, D. W. 1969. Allosteric control of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase in the marine organism Vibrio MB-22. Thesis, University of Florida, Gainesville, Florida.

115. Sanwal, B. D., M. Kapoor and H. Duckworth. 1971. The regulation of branched and converging pathways. In: Current topics in cellular regulation, B. L. Horecker and E. R. Stadtman (eds), Vol. 3. Academic Press, Inc., New York.

116. Saz, A. K., S. Watson, S. R. Brown and D. L. Lowery. 1963. Antimicrobial activity of marine waters. I. Macromolecular nature of antistaphylococcal factor. Liminol. Oceanogr. 8:63-67.

117. Scarpiro, P. V. and D. Pramer. 1962. Evaluation of factors affecting the survival of Escherichia coli in sea water. VI. Cysteine. Appl. Microbiol. 10:436-440.

118. Shapiro, B. M., H. S. Kingdom and E. R. Stadtman. 1967. Regulation of glutamine synthetase. VII. Adenyl glutamine synthetase: A new form of the enzyme with altered regulatory and kinetic properties. Proc. Natl. Acad. Sci. U.S. 58:642-649.

119. Shewan, J. M. 1963. The differentiation of certain genera of gram negative bacteria frequently encountered in the marine environments. Symp. Marine Microbiol. pp 499-521. Charles C. Thomas, Publishers, Springfield, Ill.

120. Simidu, U. and K. Hasuo. 1968. Salt dependency of the bacterial flora of marine fish. J. Gen. Microbiol. 52:347-354.

121. Simpson, R. J., B. C. Davidson, T. A. A. Dopheide, S. Andrews and J. Pittard. 1971. Purification and properties of 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthetase (phe) from a λ aro G^+ transductant of Escherichia coli. J. Bacteriol. 107:798-805.

122. Sistrom, W. R. 1960. A requirement of sodium in the growth of Rhodopseudomonas spheroides. J. Gen. Microbiol. 22:778-785.

123. Smith, L. C., J. M. Ravel, S. R. Lox and W. Shive. 1962. The control of 3-deoxy-D-arabino-heptulosonic acid 7-phosphate synthesis by phenylalanine and tyrosine. J. Biol. Chem. 237:3566-3570.

124. Sprinson, D. B. 1960. The biosynthesis of aromatic compounds from D-glucose. Advan. Carbohydrate Chem. 15:235-270.

125. Srinivason, P. E. and D. B. Sprinson. 1959. 2-keto-3-deoxy-D-arabinoheptulosonic acid 7-phosphate synthetase. J. Biol. Chem. 234:716-722.

126. Stadtman, E. R. 1963. Symposium on multiple forms of enzymes and control mechanisms. II. Enzyme multiplicity and function in the regulation of divergent metabolic pathways. Bacterio. Rev. 27:170-181.

127. _____. 1966. Allosteric regulation of enzyme activity. Adv. Enzymol. 28:41-154.

128. _____. 1970. Mechanisms of enzyme regulation in metabolism. In: The Enzymes. P. D. Boyer (ed.), Academic Press, New York. 1:397-459.

129. _____, G. N. Cohen, G. Lebras and H. Robichon-Szulmajster. 1961. Feedback inhibition and repression of aspartokinase activity in Escherichia coli and Sacchromyces cerevisiae. J. Biol. Chem. 236:2033-2038.

130. Stalon, V., F. Ramos, A. Pierard and J. M. Wiame. 1967. The occurrence of a catabolic and an anabolic ornithine carbamyltransferase in Pseudomonas. Biochim. Biophys. Acta. 139:91-97.

131. Staub, M. and G. Denes. 1967. Kinetic study of the mechanism of action of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase in Escherichia coli K-12. Biochim. Biophys. Acta. 132:528-530.

132. _____ and _____. 1969. Purification and properties of the 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase (phenylalanine sensitive) of Escherichia coli K-12. I. Purification of enzyme and some of its catalytic properties. Biochim. Biophys. Acta. 178:588-598.

133. _____ and _____. 1969. Purification and properties of the 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase (phenylalanine sensitive) of Escherichia coli K-12. II. Inhibition of activity of the enzyme with phenylalanine and functional group-specific reagents. Biochim. Biophys. Acta. 178:599-608.

134. Stern, J. R. 1967. Oxalacetate decarboxylase of Aerobacter aerogenes. I. Inhibition by avidin and requirement for sodium ion. Biochem. 6:3545-3551.

135. Strecker, H. J. 1957. Interconversion of glutamic acid and proline. I. Formation of Δ^1-pyrroline-5-carboxylic acid from glutamic acid in Escherichia coli. J. Biol. Chem. 225:825-834.

136. Takacs, F. P., T. I. Matula and R. A. MacLeod. 1964. Nutrition and metabolism of marine bacteria. XIII. Intracellular concentrations of sodium and potassium ions in a marine pseudomonad. J. Bacteriol. 87:510-518.

137. Takahashi, M. and W. C. Chan. 1971. Separation and properties of isozymes of 3-deoxy-D-arabino-heptulosonate-7-phosphate synthetase from Sacchromyces cerevisiae. Can. J. Microbiol. 49: 1015-1025.

138. Tomlinson, N. and R. A. MacLeod. 1957. Nutrition and metabolism of marine bacteria. IV. The participation of Na^+, K^+ and Mg^{++} salts in the oxidation of exogenous substrates by a marine organism. Can. J. Microbiol. 3:627-638.

139. Thompson, J., M. L. Green and F. C. Happold. 1969. Cation-activated nucleotidase in cell envelope of a marine bacterium. J. Bacteriol. 99:834-841.

140. _____ and R. A. MacLeod. 1971. Functions of Na^+ and K^+ in the active transport of α-aminoisobutyric acid in a marine pseudomonad. J. Biol. Chem. 246:4066-4074.

141. Torrianni, A. 1960. Influence of inorganic phosphate in the formation of phosphatases by Escherichia coli. Biochim. Biophys. Acta. 38:460-479.

142. Truffa-Bachi, P. and G. N. Cohen. 1966. Escherichia coli lysine-sensitive β-aspartokinase: purification and properties. Biochim. Biophys. Acta. 113:531-541.

143. Tyler, M. E., M. C. Bielling and D. B. Pratt. 1960. Mineral requirements and other characteristics of selected marine bacteria. J. Gen. Microbiol. 23:153-161.

144. Umbarger, H. E. 1956. Evidence for a negative feedback mechanism in the biosynthesis of isoleucine. Science 123:848.

145. _____. 1961. End product inhibition of the initial enzyme in a biosynthetic sequence as a mechanism of feedback control. In: Control mechanisms in cellular processes, D. M. Bonner (ed.), pp 67-85. Ronald Press, New York.

146. _____. 1961. Feedback control by end product inhibition. Cold Spring Harbor Symp. Quant. Biol. 26:301-312.

147. _____. 1969. Regulation of amino acid metabolism. Ann. Rev. Biochem. 38:323-370.

148. _____, and B. Brown. 1957. Threonine deamination in Escherichia coli. II. Evidence for two L-threonine deaminases. J. Bacteriol. 73:105-112.

149. _____ and _____. 1958. Isoleucine and valine metabolism in Escherichia coli. VII. A negative feedback mechanism controlling isoleucine biosynthesis. J. Biol. Chem. 233:415-420.

150. _____ and _____. 1958. Isoleucine and valine metabolism in Escherichia coli. VIII. The formation of acetolactate. J. Biol. Chem. 233:1156-1160.

151. _____ and B. D. Davis. 1962. Pathways of amino acid biosynthesis. In: The Bacteria. I. C. Gunsalas and R. Y. Stanier (eds.), Academic Press, New York. 3:167-241.

152. Wallace, B. J. and J. Pittard. 1967. Genetic and biochemical analysis of the isozymes concerned in the first reaction of aromatic biosynthesis in Escherichia coli. J. Bacteriol. 93: 237-244.

153. _____ and _____. 1967. Chromatography of 3-deoxy-D-arabino-heptulosonate 7-phosphate synthetase (trp) on DEAE cellulose: a correction. J. Bacteriol. 94:1279.

154. Webb, C. D. and W. J. Payne. 1971. Influence of Na^+ on synthesis of macromolecules by a marine bacterium. Appl. Microbiol. 21:1080-1088.

155. Weber, H. L. and A. Böck. 1968. Comparative studies on the regulation of DAHP synthetase activity in blue-green and green algae. Archiv. Mikrobiol. 61:159-168.

156. Westlake, D. W. S., D. F. Horler and W. B. Connell. 1967. The effect of sodium on the fermentation of glutamic acid by Peptococcus aerogenes. Biochem. Biophys. Res. Commun. 26:461-465.

157. Woolfolk, C. A. and E. R. Stadtman. 1964. Cumulative feedback inhibition in the multiple end product regulation of glutamine synthetase activity in Escherichia coli. Biochem. Biophys. Res. Commun. 17:313-319.

158. _____ and _____. 1967. Regulation of glutamine synthetase: III. Cumulative feedback inhibition of glutamine synthetase from Escherichia coli. Arch. Biochem. Biophys. 118: 736-755.

159. Wong, P. T. S., J. Thompson and R. A. MacLeod. 1969. Nutrition and metabolism of marine bacteria. XVII. Ion-dependent retention of α-aminoisobutyric acid and its relation to Na^+-dependent transport in a marine pseudomonad. J. Biol. Chem. 244:1016-1025.

160. Yates, R. A. and A. B. Pardee. 1956. Control of pyrimidine biosynthesis in Escherichia coli by a feedback mechanism. J. Biol. Chem. 221:757-770.

161. Zobell, C. E. and H. C. Uppham. 1944. A list of marine bacteria including descriptions of sixty new species. Bull. Scripps Inst. Oceanogr. 5:239-292.

BIOGRAPHICAL SKETCH

Andrew Mark Chludzinski was born September 15, 1945, in Gdynia, Poland. His family immigrated to the United States in 1946. In June, 1962, he was graduated from Brooklyn Technical High School. In June, 1966, he received the degree of Bachelor of Science in Biology from St. John's University. From September, 1966, until the present time he has pursued work toward the degree of Doctor of Philosophy in the Department of Microbiology.

Andrew Mark Chludzinski is married to the former Margaret Mary Schwarzkopf. They are the proud parents of twin boys, Michael Andrew and Richard Mark. The author is a member of the American Society for Microbiology.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

DeLill S. Nasser, Chairman
Associate Professor of Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Eugene G. Sander
Associate Professor of Biochemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Edward P. Previc
Associate Professor of Microbiology

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Max E. Tyler
Professor of Microbiology

This dissertation was submitted to the Dean of the College of Agriculture and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

August, 1972

John E. Reynolds
Asst. Dean, College of Agriculture

Dean, Graduate School